IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Hu et al.

Application No.: 10/670,971 Examiner: Nguyen, Quang

Filed: September 24, 2003 Group Art Unit: 1636

For: EPIDERMAL MELANOCYTE CULTURE FORMULATIONS

DECLARATION OF DAN-NING HU
UNDER 37 C.F.R. § 1.132

I hereby certify that this paper is being deposited with the United States Postal Service as First Class mail in an envelope addressed to: Mail Stop Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on November 16, 2004.

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November 16, 2004
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Commissioner for Patents
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Alexandria, VA 22313-1450

I, DAN-NING HU, hereby declare as follows:

1. I am a co-inventor of the invention disclosed and claimed in the present United States patent application.

2. I hold the following relevant positions: (1) Director, Tissue Culture Center, New York Eye and Ear Infirmary; (2) Research Professor of Ophthalmology, New

York Medical College; (3) Chairman, Society of Ocular Pigment Cell Research; (4) Chairman, Ocular / Extracutaneous Pigmentation Interest Group, International Federation of Pigment Cell Research; and (5) Associate Editor, Pigment Cell Research.

3. I have reviewed the Office Action mailed by the U.S. Patent and Trademark Office on June 16, 2004 for the present application. I have also reviewed Swope et al., 1995, Experimental Cell Research 217:453-459 ("Swope"), U.S. Patent No. 5,916,809 to Yanase et al., 1994 ("Yanase"), Chen et al, 2000, J. Dermatology 27:434-439 ("Chen"), Hu et al., 2000, Exp. Eye Res. 71:217-224 ("HuI"), and Hu, 2000, Pigment Cell Res. 13 Suppl. 8:81-86 ("HuII"). Each of these references was cited by the Examiner in the rejections of the claims as set forth in the June 16, 2004 Office Action.

4. I provide this declaration, in part, to explain why, with the Swope, Yanase, Chen, HuI and HuII references at hand (each of which pertain only to uveal melanocytes), a person of ordinary skill in the art nevertheless would understand that uveal melanocytes are radically different from epidermal melanocytes ("EM") and, therefore, would not have had a reasonable expectation that culture formulations for growing uveal melanocytes would be useful for culturing EM.

5. Hu's paper (1) studied uveal melanocytes which are located in the eye, and which are distinctly different from the epidermal melanocytes (EM) located in skin. EM and uveal melanocytes are entirely different cells, each with an entirely different cell biology and patho-physiology.

6. A partial listing of the great differences between EM and uveal melanocytes, which would have been appreciated by a person of ordinary skill in the art, are as follows:

a) EM respond to ultraviolet radiation (e.g., tan color of skin) but uveal melanocytes do not (1, 2, 3).

b) EM transfer melanin to keratinocytes but uveal melanocytes do not transfer melanin (1, 2, 3).

c) EM respond to many inflammatory diseases by the changing of skin color but this is very rare in the eye (1, 2).

d) The malignant transformation of EM, cutaneous melanoma, increases in incidence by ultraviolet exposure over a period of years, but uveal melanoma exhibits no such increase (4, 5).

e) Metastasis of skin melanoma is mainly through the lymphatics - skin metastasis is common. Metastasis of uveal melanoma is mainly through the blood vessels, leading to the liver (6).

f) Cytogenetics studies: Skin melanoma exhibits changes of chromosome 1, 6, 7, 9 and 10. Uveal melanoma exhibits changes of chromosome 3, 6 and 8 (6, 7).

g) Molecular genetics: Skin melanoma most commonly demonstrates RAS and BRAF mutations and ß-catenin is mutated in 25% cases. Uveal melanoma demonstrates no RAS, BRAF and ß-catenin mutations (6, 8).

h) Integrin expression: Skin melanoma expresses α2 commonly and α5 rarely. Uveal melanoma exhibits α2 rarely and α5 commonly (9).

i) In vitro: EM respond to ACTH and α-MSH but uveal melanocytes do not (1).

j) Uveal melanocytes respond to β2 (but not β1) adrenoreceptor agonist (10); therefore, based on this information, a person of ordinary skill in the art would use a specific β2 agonist. Surprisingly, epinephrine has a significantly greater effect on EM than demonstrated by either specific β1 or β2 adrenoreceptor agonists.

7. In light of the distinct cytology of EM and uveal melanocytes, therefore, the effect of any compounds on EM growth, melanogenesis, and migration must be tested individually with no expectation that any single compound (or combination), which was useful for culturing uveal melanocytes, would be suitable for EM growth, melanogenesis, and migration.

8. For example, we selected epinephrine for culturing EM rather than a specific adrenergic agonist only after we undertook a separate study of the effects of several adrenergic agonists on EM and uveal melanocytes. In uveal melanocytes, only the β2 adrenoreceptor agonist, metaproterenol, was found to stimulate their growth and melanogenesis. Notably, a β1 adrenoreceptor agonist (prenalterol) and a β3 adrenoreceptor agonist (D-7114) did not have any effect on uveal melanocyte growth and melanogenesis (10) indicating that uveal melanocytes were specifically responsive to β2 adrenoreceptor agonists.

9. Accordingly, with knowledge of the response of uveal melanocytes to the various adrenoreceptor agonists discussed above, a person of ordinary skill in the art - IF looking to add an adrenoreceptor agonist to a culture medium formulation - would have selected a specific β2 adrenoreceptor agonist rather than the non-selective agonist, epinephrine.

10. In reality, the responsiveness of EM to the various adrenoreceptor agonists was quite different than that of uveal melanocytes. Both β1 (prenalterol) adrenoreceptor agonist and β2 adrenoreceptor agonist (metaproterenol) were found to stimulate the growth and melanogenesis of EM. Importantly, the effects of epinephrine were surprisingly much more potent than that of the β2 adrenoreceptor agonist, further indicating that the mechanism of action of adrenergic agonists is different in EM and uveal melanocytes.

11. These results are shown in the enclosed bar graphs depicting growth (**Figure 1**) and melanogenesis (**Figure 2**) of EM in the presence of epinephrine (bar 2), a specific β1 adrenoreceptor agonist (bar 3 labeled beta-1), a specific β2 agonist (bar 4 labeled beta-2), and a specific β3 agonist (bar 5 labeled beta-3). These data were obtained in accordance with the methods disclosed in the present specification. **Figure 1** shows the effects of the various adrenergic agonists on cell growth of cultured EM. Cells were cultured with cAMP-elevating agents-deleted culture medium (control) or supplemented with the various adrenergic agonists. Epinephrine, prenalterol (a β1 adrenoreceptor agonist), metaproterenol (a β2 adrenoreceptor agonist), and D-7114 (a β3 adrenoreceptor

agonist) were added to the culture medium at concentration of 10^{-5} M. Cells were cultured for six days, after which cell number was counted and compared. The results are expressed as percentages of controls (three wells per group, Mean ± SD). Figure 1 clearly demonstrates that the addition of epinephrine, metaproterenol and prenalterol induces a significantly greater cell number than that of the control ($p < 0.01$). Cell number of EM cultured with D-7114 showed no significant difference as compared with the control ($p > 0.05$). Cell number in EM cultured with epinephrine were significantly greater than that of EM cultured with metaproterenol ($0.05 > p > 0.01$) or prenalterol ($p < 0.01$). **Figure 2** shows the effects of various adrenergic agonists on melanogenesis in cultured EM. Cells were cultured with cAMP-elevating agents-deleted culture medium (control) or supplemented with the various adrenergic agonists. Epinephrine, prenalterol (a β1 adrenoreceptor agonist), metaproterenol (a β2 adrenoreceptor agonist), and D-7114 (a β3 adrenoreceptor agonist) were added to the culture medium at concentration of 10^{-5} M. Cells were cultured for six days, after which the amount of melanin per well was measured and compared. The results are expressed as a percentage of control (three wells per group, Mean ± SD). Figure 2 demonstrates that the addition of epinephrine, metaproterenol and prenalterol induces a significantly greater melanin content per well than that of the control ($p < 0.01$). The melanin content per well measured in EM cultured with D-7114 showed no significant difference as compared to control ($p > 0.05$). The melanin content per well in EM cultured with epinephrine was significantly greater than that of EM cultured with metaproterenol ($0.05 > p > 0.01$) or prenalterol ($p < 0.01$).

12. The references cited herein are listed below. Copies of these references are enclosed for the Examiner's convenience.

1. Hu DN. Regulation of growth and melanogenesis of uveal melanocytes. Pigment Cell Res 2000; 13 (Suppl. 8): 81-86.

2. Boissy RE. The melanocytes: its structure, function, and subpopulations in skin, eye and hair, Dermatologic Clinics 1988; 6: 161-173.

3. Boissy RE. Extracutaneous melanocytes, in *The Pigmentary System*, Edited by Nordland JE et al. Oxford Univ. Press, Oxford, 1998. p. 68.

4. Singh AD, Rennie IG, Seregard S, Giblin M, McKenzie J. Sunlight exposure and pathogenesis of uveal melanoma. Surv. Ophthalmol. 2004; 49, 419-28.

5. Bergman L, Seregard S, Nilsson B, Ringborg U, Lundell G, Ragnarsson-Olding B. Incidence of uveal melanoma in Sweden from 1960 to 1998. Invest. Ophthalmol. Vis. Sci. 2002; 43, 2579-2583.

6. Edmunds SC, Kelsell DP, Hungerford JL, Cree IA. Mutational analysis of selected genes in the TGFβ, Wnt, pRb, and p53 pathways in primary uveal melanoma. Invest. Ophthalmol. Vis. Sci. 2002: 43: 2845-2851.

7. Loercher A.E, Harbour JW. Molecular genetics of uveal melanoma. Curr. Eye Res. 2003; **27**, 69-74.

8. Cohen Y, Goldberg-Cohen N, Parrella P, Chowers I, Merbs SL, Pe'er J, Sidransky D. Lack of BRAF mutation in primary melanoma. Invest. Ophthalmol. Vis. Sci. 2003, 44: 2876-2878.

9. ten Berge PJ, Danen EH, van Muijen GN, Jager MJ, Ruiter DJ. Integrin expressions

in uveal melanoma differs from cutaneous melanoma. Invest Ophthalmol Vis Sci 1993; 34:3635-3640.

10. Hu DN, Woodward DF, McCormick SA. Influence of autonomic neurotransmitters on human uveal melanocytes in vitro. Exp Eye Res 2000; 71:217-24.

13. In conclusion, it is my opinion that a scientist in the field of melanocytology would not reasonably have been expected to succeed in making a formulation for culturing epidermal melanocytes, as claimed in the present application, with the experience of using culture formulations for uveal melanocytes. Indeed, if at all motivated to include an adrenergic agonist in the formulation (aware of potential undesired interactions with other components of the formulation), the scientist in the field would have selected a specific β2 adrenoreceptor agonist and not the non-specific agonist, epinephrine.

14. Moreover, the prior art also has recognized that the scientific community has long been searching for a medium suitable for culturing melanocytes to produce melanocytes that could be successfully transplanted. Thus, given this general belief in the research community, in addition to the myriad of unpredictable factors in creating such a culture medium, one could not fairly expect that a combination of findings obtained from studies in uveal melanocytes would lead a person of ordinary skill in the art to make the formulation that is claimed in the present application.

15. I hereby declare that all statements made herein of my own

knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of any patent issuing from the above-captioned patent application.

Nov. 15, 2004
Date

DAN-NING HU

PIGMENT CELL RES 13 (Suppl. 8): 81–86. 2000
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ISSN 0893-5785

Keynote Lecture

Regulation of Growth and Melanogenesis of Uveal Melanocytes

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Received 24 December 1999; in final form 8 February 2000

We have developed methods for the isolation, cultivation, and investigation of human uveal melanocytes (UM). Uveal melanocytes grow well and produce melanin in vitro in the presence of basic fibroblast growth factor (bFGF), cyclic adenosine monophosphate-elevating agents, and serum. Cultured UM respond to various factors. Certain growth factors (bFGF and hepatocyte growth factor, etc.), endothelin, adrenergic β_2-receptor agonists, and some prostaglandins (EP_2-receptor agonists and certain TP-receptor agonists) stimulate, while transforming growth factor-β_2, interleukin-6, and cholinergic agonists inhibit melanogenesis and/or growth of UM in vitro. α-Melanocyte-stimulating hormone, adrenocorticotropic hormone, various sex hormones, and prostaglandin $F_{2\alpha}$ showed no effect on the growth and melanogenesis of cultured UM. The stability of UM in vivo may be controlled by these factors. Disturbance of this balance may lead to certain rare pathologic pigmentary changes of the iris. UM are relatively stable in vivo; they usually do not respond (proliferate or show dynamic changes in melanogenesis) to various environmental factors. The differences of the in vivo behavior between uveal and epidermal melanocytes may be determined by both cellular factors and environmental factors.

Key words: Prostaglandin, Cell culture, Hormone, Neurotransmitter, Growth factor

INTRODUCTION

There are two types of ocular pigment cells: pigment epithelial cells, which originate from the neural ectoderm, and uveal melanocytes (UM), which are derived from the neural crest. The melanocytes show a limited response to injury and inflammation, but they do undergo neoplastic transformation. Uveal melanoma is the most common intraocular malignant tumor (1, 2). By contrast, the pigment epithelia respond to trauma and inflammation by proliferation, but neoplasms of the pigment epithelia are very rare (2). Both cell types have been isolated, cultured, and studied in vitro. This study will concentrate on the study of UM in vitro.

The UM differ from the epidermal melanocytes in certain aspects, mainly: 1) the epidermal melanocytes synthesize and transfer melanin to keratinocytes, while UM do not transfer melanin to other cells in vivo; 2) the epidermal melanocytes grow in vivo and respond to UV radiation, injury, and inflammation. Changes of skin color due to environmental factors occur in many physiologic or pathologic situations. The UM are relatively stable in vivo; they usually do not respond to various environmental factors and pigmentary changes of the iris are not common (1–3).

There are two possible explanations for these differences between these two cells: 1) UM are different from the epidermal melanocytes – they do not respond to various factors, such as growth factors, neurotransmitters, inflammatory mediators, and hormones in vivo and in vitro; or 2) the environment of the UM (the eye) is different from that of the epidermal melanocytes (the skin).

Until approximately one decade ago, it was impossible to study these hypotheses. In 1980s, methods for studying melanocytes in vitro were established for epidermal

Abbreviations – UM: uveal melanocytes; IBMX: isobutylmethylxanthine; TPA: 12-o-tetradecanoyl-phorbol-13-acetate; dbcAMP: dibutyryl-cAMP; PG: prostaglandin; bFGF: basic fibroblast growth factor

melanocytes, but not for UM (4–9). Studies of UM have therefore lagged behind.

Since 1990, we have developed methods for the isolation, cultivation, and investigation of UM. Many pure cell lines of human UM and the in vitro model for studying the regulation of the growth and melanogenesis of UM have been established (10–12). Many growth factors, cytokines, hormones, neurotransmitters, prostaglandins, and other factors have been tested for their effects on the UM (11, 13–16). Now, the gaps between the knowledge of regulation of growth and melanogenesis of these two cells are narrowing. Furthermore, many biologic factors, mainly various neurotransmitters and prostaglandins, have been first studied extensively in the UM (16, 17). This study is a brief summary of the studies on regulation of growth and melanogenesis of UM performed in our laboratory.

MATERIALS AND METHODS

Isolation and Culture of UM

Human UM were isolated from donor eyes using methods we developed, as previously described (10). Briefly, the uvea was isolated from the sclera and retina. The pigment epithelium was detached from the uvea by an enzymatic dissociation method (trypsin), and then the UM were isolated by our trypsin–collagenase sequential method.

The isolated UM were cultured with one of the following culture media: 1) FIC medium: F-12 medium supplemented with basic fibroblast growth factor (bFGF; 20 ng/ml), isobutylmethylxanthine (IBMX; 0.1 mM), cholera toxin (10 ng/ml), 10% fetal bovine serum (FBS), glutamine (2 mM), and gentamicin (50 μg/ml). 2) TIC medium: FIC medium with bFGF replaced by 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA, 50 ng/ml). 3) TI medium: TIC medium with a deletion of cholera toxin.

The long-term results of UM cultured with these three media have been compared in three cell lines. In each cell line, the UM were seeded into three flasks at the first subculture and cultured separately with 3 different media. Cells were incubated and subcultured continuously until senescence. The cumulative population doublings of each cell line cultured with these three media were calculated as previously described (10).

Ten cell lines of UM isolated from donors with different iris color were studied for melanogenesis of UM. Methods for ultrastructural study and measurement of melanin content, melanin production, and tyrosinase activity have been described previously (12).

Experiments

The effects of various agents on the growth and melanogenesis of UM were tested by substitution studies, as described previously (11, 13). Briefly, early passages of UM were seeded into multi-well plates with FIC medium. After 24 hr, the medium was replaced by test media. Various test substances at different concentrations were added to the media. Cells cultured with test media without the addition of test substances were used as controls. Each group was tested in triplicate. The media were replaced every 3 days. After 6 days, the cells were detached for cell counting and melanin measurement, as previously described (11–13). A Student's *t*-test was used to assess statistical significance.

Four different media were used to test the effects of various substances: 1) complete medium: FIC medium; 2) bFGF/TPA-deleted medium: FIC medium with a deletion of bFGF; 3) cyclic adenosine monophosphate (cAMP)-deleted medium: FIC medium with a deletion of cAMP-elevating agents (IBMX and cholera toxin); 4) serum-deleted medium: FIC medium without serum.

RESULTS

UM isolated and cultured using our methods grew well in vitro. A comparative study for testing the effects of three media showed that the average cumulative population doublings for cells cultured with FIC medium, TIC medium, and TI medium were 32.5, 30.6 and 28.0, respectively. Cells cultured with FIC medium grew better than those in other media. However, the differences were not statistically significant. Many pure cell lines of UM have been established, most of them grown in FIC medium. UM grow very well in FIC medium, with a doubling time of 24–48 hr. They can divide 35–50 times in vitro over a 3–6 month period.

Cultured UM express tyrosinase activity, produce measurable melanin, and maintain a constant level of melanin content per cell in vitro. Numerous immature melanosomes are present in the cytoplasm. UM from dark-colored irides produce more melanin than those from light-colored irides. These results indicate that cultured UM can be a good in vitro model system for studying the melanogenesis of UM (12).

The effects of various agents on the growth and melanogenesis of UM were:

1. Growth factors and cytokines: bFGF-stimulated growth of cultured human UM. The stimulating effects were dose-dependent at concentrations of 1–100 ng/ml. Hepatocyte growth factor (HGF), fibroblast growth factor-6 (FGF-6), and keratinocyte growth factor (KGF) showed similar but less stimulating effects on UM in bFGF/TPA-deleted medium. Other growth factors, such as epidermal growth factor (EGF), nerve growth factor (NGF), platelet-derived growth factor AB (PDGF), vascular endothelial growth factor (VEGF), and acidic fibroblast growth factor (aFGF) did not have any effects on the growth and melanogenesis of UM in bFGF/TPA-deleted medium at a concentration from 1–100 ng/ml. Transforming growth factor-β2 (TGF-β2) showed marked inhibitory effects on the growth of UM (0.03–10 ng/ml). Interleukin-6 (IL-6) had mild inhibitory effects on the growth and melanogenesis of UM (Table 1).
2. Hormones: α-melanocyte-stimulating hormone (α-MSH) (10^{-5}–10^{-8} M), adrenocorticotropic hormone (ACTH), progesterone, and estradiol (0.1–10 μg/ml) did not have significant effects on the growth and melanogenesis of UM in cAMP-deleted medium. Melatonin showed no effects on the growth and melanogenesis of UM in complete medium at concentrations of 10^{-10}–10^{-7} M. Endothelin 1 stimulated the growth and melanogenesis

Table 1. Effect of growth factors, cytokines and hormones on growth and melanogenesis of cultured human uveal melanocytes

Substance	Test media[1]	Growth[2]	Melanogenesis[2]
BFGF	BD	++	0
HGF	BD	++	0
FGF-6	BD	++	0
KGF	BD	+	0
aFGF	BD	0	0
EGF	BD	0	0
NGF	BD	0	0
PDGF	BD	0	0
VEGF	BD	0	0
TGF-β2	C	--	-
IL-6	C	-	-
α-MSH	CD	0	0
ACTH	CD	0	0
Progesterone	CD	0	0
Estradiol	CD	0	0
Endothelin 1	CD	++	+
Melatonin	C	0	0

[1]Test media: C, complete medium; BD, bFGF/TPA-deleted medium; CD, cAMP-deleted medium. [2]Effects: +, significantly higher than the controls; ++: significantly higher and more than 200% of the controls; 0, no significant difference compared with the controls; -, significantly lower than the controls; --, significantly lower and less than 50% of the controls.

of UM in cAMP-deleted medium at concentrations of 10^{-10}–10^{-6} M (Table 1).

3. Neurotransmitters: the adrenergic agonist, epinephrine, which activates both α-adrenergic and β-adrenergic receptors, stimulated the growth and melanogenesis of cultured UM in cAMP-deleted medium. Methoxamine and clonidine, which activate α_1-adrenergic and α_2-adrenergic receptors, showed no effects. Isoproterenol, which activates β_1-receptors and β_2-receptors, stimulated the growth and melanogenesis of cultured UM in cAMP-deleted medium (10^{-9}–10^{-5} M). The β_2-receptor agonists, metaproteronal and salbutamol, also showed stimulating effects, but the β_1-receptor and β_3-receptor agonists (metaproteronal and D-7114) did not have any effects. The cholinergic agonist, muscarine, inhibited the growth and melanogenesis of UM in complete and serum-deleted medium (Table 2).
4. Prostaglandins (PGs): PGE_1, PGE_2, PGD_2, PGI_2, PGA_2, and one of the TP-receptor agonists (AGN 192093) stimulated the growth and melanogenesis of UM in cAMP-deleted medium at relativly high concentrations (10^{-5} M, PGI_2 at 10^{-4}-M level). The EP_2-receptor agonist AH13205 also showed stimulating effects (10^{-6}–10^{-5} M), but the EP_1 and EP_3-receptor agonist (sulprostone) did not have any effects in cAMP-deleted medium. The IP-receptor agonists (cicaprost and iloprost), DP-receptor agonist (BW 245C), and one of the TP-receptor agonists (U-46619) did not have effect (Table 3). $PGF_{2\alpha}$ and its analog latanoprost and

Table 3. Effect of PGs and other substances on growth and melanogenesis of cultured human uveal melanocytes

Substance	Tested media[1]	Growth[2]	Melanogenesis[2]
PGA_2	CD	+	+
PGD_2	CD	+	+
PGE_1	CD	+	+
PGE_2	CD	+	+
$PGF_{2\alpha}$	C, CD, BD, SD	0	0
PGI_2[3]	CD	+	+
BW 245C (DP agonist)	CD	0	0
Sulprostone (EP_1, EP_3 agonist)	CD	0	0
AH 132052 (EP_2 agonist)	CD	++	++
Cicaprost (IP agonist)	CD	0	0
Iloprost (IP agonist)	CD, BD	0	0
AGN 192093 (TP agonist)	CD, BD	+	+
U-46619 (TP agonist)	CD, BD	0	0
Latanoprost (FP agonist)	C, CD, BD, SD	0	0
PhXA85 (FP agonist)	C, CD, BD, SD	0	0
TPA	BD	++	++
IBMX	CD	++	++
dbcAMP	CD	++	++
Cholera toxin	CD	+	+
κ-Casein	C	--	--

[1]Test media: C, complete medium; BD, bFGF/TPA-deleted medium; CD, cAMP-deleted medium; SM, serum-deleted medium. [2]Effects: +, significantly higher than the controls; ++, significantly higher and more than 200% of the control; 0, no significant difference as compared with the controls; --, significantly lower and less than 50% of the controls. [3]PGI_2 stimulates growth and melanogenesis of cultured UM only at very high concentration (10^{-4} M).

Table 2. Effect of neurotransmitters on growth and melanogenesis of cultured human uveal melanocytes

Substance	Receptors	Test media[1]	Growth[2]	Melanogenesis[2]
Epinephrine	α-, β-adrenergic	CD	++	+
Phenylephrine	α-, β-adrenergic[3]	CD	+	+
Methoxamine	α_1-adrenergic	CD BD	0	0
Clonidine	α_2-adrenergic	CD BD	0	0
Isoproterenol	$\beta_{1,2,3}$-adrenergic	CD	++	++
Prenalterol	β_1-adrenergic	CD	0	0
Metaproterenol	β_2-adrenergic	CD	++	++
Salbutamol	β_2-adrenergic	CD	++	++
D-7114	β_3-adrenergic	CD	0	0
Muscarine	Cholinergic	C, SD	-	-

[1]Test media: C, complete medium; BD, bFGF/TPA-deleted medium; CD, cAMP-deleted medium; SM, serum-deleted medium. [2]Effects: +, significantly higher than the controls; ++, significantly higher and more than 200% of the control; 0, no significant difference as compared with the controls; -, significantly lower than the controls. [3]Phenylephrine preferentially activates α_1-adrenergic receptors but also stimulates β-adrenergic receptors at high concentrations.

PhXA85 did not affect the growth and melanogenesis of cultured iridal melanocytes from blue, green, and brown irides and one cell line from mixed-colored iris in all four tested media (Table 3).

5. Other factors: TPA stimulates the growth and melanogenesis of UM in bFGF-deleted medium (1–100 ng/ml). Three cAMP-elevating agents, IBMX, dibutyryl-cAMP (dbcAMP), and cholera toxin stimulated the growth and melanogenesis of UM in cAMP-deleted medium. κ-Casein, a protein in milk, inhibited both growth and melanogenesis in UM at very high concentrations (0.1–1.0 mg/ml) (Table 3).

DISCUSSION

Various biologic substances may stimulate or inhibit the growth and melanogenesis of UM. To test the stimulating effects of various substances, we developed a substitution model for these investigations (11, 13). The principle is as follows: when one of the known essential factors is deleted from complete medium, melanogenesis and/or growth of UM is inhibited. A test substance is added to the 'deleted' media. If the test substance has an effect similar to the deleted agent, then the growth and melanogenesis of the UM will recover. In our previous study, we found that three groups of factors, bFGF or TPA, various cAMP-elevating agents, and serum are essential for the growth, melanogenesis, and survival of UM in vitro (11, 13). Deletion of any one of these factors from the complete medium resulted in three different deleted media, the bFGF/TPA-deleted medium, cAMP-deleted medium, and serum-deleted medium. Based on the known mechanism of the effects of the test substance, one or more deleted media can be selected as the test media. To test inhibitory effects, complete medium or other media can be used as the test media.

In the study of the melanogenesis of UM, three parameters (melanin content per cell, melanin content per culture, and tyrosinase activity) were used to evaluate the effect of various substances on the melanogenesis of cultured UM (12, 13). An important concept for consideration is which parameter is most meaningful in the evaluation of the melanogenesis of UM in vitro. Tyrosinase activity is an important factor, but not the sole factor in determining the rate of melanin production (18). The color of the iris is determined by melanin content rather than tyrosinase activity (12). Measurement of melanin content is more direct and meaningful than that of tyrosinase activity. Melanin content per culture is more important than melanin content per cell for two reasons. First, melanin content per cell is affected both by the melanin production rate and the growth rate. In stationary cells, melanin produced accumulates in the cell and results in a rapid increase in the melanin content per cell. In growing cells, melanin is diluted to daughter cells during division and results in a marked decrease of melanin per cell (13). Furthermore, from the clinical viewpoint, pigmentation is a function of the total melanin present in the tissue, not of melanin content per melanocyte (13). Therefore, melanin content/culture was used as the main parameter for evaluating the effects of various substances on the melanogenesis of cultured UM.

In this study, we found that many factors regulate the growth and melanogenesis of cultured UM. Some of them deserve further discussion. Among the hormones, the most interesting finding is that the α-MSH and ACTH do not have significant effects on the growth and melanogenesis of UM in cAMP-deleted medium, while they usually show stimulating effects on the growth and melanogenesis of epidermal melanocytes (19–22). Boissy obtained similar results and found that the receptors for α-MSH (melanocortin-1 receptor) are absent in the UM (23). However, controversy still exists concerning the existence of melanocortin-1 receptors in the UM (24, 25). Melatonin does not have effects on growth and melanogenesis of UM in vitro; however, melatonin receptors have been detected in the UM (26). It is not known that these receptors are without function or have an effect on functions other than those we have tested.

The effects of neurotransmitters on melanocytes have been studied extensively and systematically, first in the UM. This study indicates that adrenergic agonists stimulate the growth and melanogenesis of UM in vitro; this effect is mainly through the adrenergic β_2-receptors. Zhao (17) reported similar results and demonstrated the presence of β-adrenergic receptors in the UM by immunoblotting methods. The cholinergic agonist, muscarine, inhibits the growth and melanogenesis of UM in vitro. Zhao found that another cholinergic agonist, acetylcholine, also had inhibitory effects (17). These studies indicate that the growth and melanogenesis of UM are modulated by reciprocal innervation. Adrenergic agonists stimulate while cholinergic agonists inhibit the growth and melanogenesis of UM.

Many PGs have been studied in this study. PGE_1, PGE_2, and PGA_2 stimulate the growth and melanogenesis of cultured UM in cAMP-deleted medium. The EP_2-receptor agonist shows stimulating effects, but the EP_1 and EP_3-receptor agonists do not. Therefore, the PGEs and PGA_2 may stimulate the UM through the activation of EP_2 receptors. This result is consistent with the known function of EP_2 receptors (27) and the increase in iris pigmentation of monkey eyes following local application of PGE_2, a natural EP_2 agonist (28). PGD_2 and PGI_2 (only in high levels) also stimulate the growth and melanogenesis of UM in vitro, but the DP and IP-receptor agonists do not have any effects. The underlying mechanism of these effects of PGD_2 and PGI_2 requires further investigation.

Latanoprost, a $PGF_{2\alpha}$ analog and an anti-glaucoma drug, causes iris pigmentation in about 10% of glaucoma patients (29). But $PGF_{2\alpha}$, latanoprost, and PhXA85 (the active form of latanoprost) did not stimulate the melanogenesis and growth of cultured UM in this study. This result is consistent with other reports that $PGF_{2\alpha}$ and PhXA85 had no effect on the cell number of cultured UM and ocular melanoma cells (3). The explanation may be: 1) clinical observations indicate latanoprost-induced iris pigmentation mainly occurs in patients with mixed colored irides (29). It is possible that latanoprost only selectively stimulates the iridal melanocytes from mixed colored irides. In another series of studies we found that latanoprost increased the transcription of the tyrosinase gene in iridal melanocytes from the mixed color iris, but not in those from blue and brown irides

(30); 2) the effect of $PGF_{2\alpha}$ may be indirect. For example, it may stimulate other types of cells to produce some substances that stimulate melanogenesis in UM, or it may activate UM to respond to some substances that normally do not have melanogenic activity.

This study indicates that UM respond to many biologic factors. These factors can be divided into two categories: stimulators, which include some growth factors (bFGF, HGF, FGF-6 and KGF), β2-adrenergic agonists, endothelin, and certain PGs; and inhibitors, which include TGF-β, IL-6, and cholinergic agonists. The stability of UM in vivo is controlled by these factors. The disturbance of this balance may lead to the rare pathologic pigmentary changes of the iris, such as depigmentation of the iris due to paralysis of the sympathetic nerve (absence of adrenergic stimulation).

When the effects of various factors on the UM are compared with those of epidermal melanocytes, we found that the response of UM to these factors is similar to that of epidermal melanocytes. The main exception is the lack of response of UM to α-MSH and ACTH. α-MSH and ACTH stimulate the growth and melanogenesis of cultured human epidermal melanocytes in cAMP-deleted medium, and the receptor of α-MSH (melanocortin-1 receptor) is considered a key regulator of human cutaneous pigmentation (19–21, 31).

The present studies indicate that the difference in in vivo behaviors between UM and epidermal melanocytes is determined by both cellular factors and environmental factors. 1) Cellular factors: epidermal melanocytes respond to αMSH and ACTH, but UM do not. 2) Environmental factors: many factors may influence the environment of melanocytes, such as the blood–aqueous barrier in the eye, which restricts the entering of high molecular substances from serum to the aqueous humor and may limit the growth capacity of UM (32). A high level of TGF-β2 is present in the aqueous humor and ocular tissues. TGF-β2 at these levels inhibits the growth of cultured UM. Therefore, TGF-β may be one of the factors that maintain the stability of UM in vivo (14). Furthermore, in the skin, keratinocytes directly communicate and send growth factors to epidermal melanocytes, but there are no keratinocytes in the eye.

Studies of the cultured UM in past decades have led to a better understanding of the regulation of the growth and melanogenesis of UM. However, many aspects of the UM still require further investigation. For example, our previous report indicates that the pigment epithelium contains mainly eumelanin, but that UM contain both eumelanin and pheomelanin. The color of the iris may be determined by both the amount and the type of melanin. In cultured UM, the pheomelanin/eumelanin ratio is higher in actively growing cells than in senescent cells (33). The detail of eumelanin/pheomelanin variance in UM from various parts of the eye and from eyes with different colors of irides and the significance of these findings require further study.

Secondly, little is known about the metabolic pathways of melanin in the eye. We have collaborated with Seth Orlow, using immunocytochemical methods to detect various enzymes related to melanogenesis in cultured human UM. Most of the known enzymes and substances involved in the pathway of melanogenesis have been detected in UM, such as tyrosinase, TRP-1, TRP-2, Silver protein and Pink-eye diluted protein (unpublished observation). However, UM may contain some enzymes that are not present in the skin, because in ocular albinism, depigmentation occurs only in the eye, not in the skin.

Lastly, but not of the least importance, is the physiologic function of ocular pigment cells. It has been reported that the ocular pigment epithelium, mainly the RPE, possesses many functions unrelated to melanin, such as phagocytosis of the outer segment of photoreceptors, metabolism of vitamin A, transport of water and ions, production of extracellular matrix, growth factors, cytokines, and neurotrophic factors; these functions are essential for the visual process. The RPE also play an important role in the pathogenesis of many eye diseases (34, 35). It will be of interest to study the functions of the UM and their role in the pathogenesis of eye diseases.

Acknowledgements – Supported by the New York Eye and Ear Infirmary Pathology Research Fund and the Department of Ophthalmology Research Fund. The author thanks the collaborators in the study of ocular pigment cells, including Drs. Guiseppe Prota (Italy), Seth Orlow (New York), Shosuke Ito (Japan), Joan E. Roberts (New York), David F. Woodward (California), and Johan Stjernschantz (Sweden).

REFERENCES

1. Naumann GOH, Apple DJ. Pathology of the Eye. New York: Spring-Verlag; 1986. 430 p
2. Yanoff M, Fine BS. Ocular Pathology: A Text and Atlas, 4th edition. London: Mosby-Wolfe; 1996. 614 p
3. Imesch PD, Wallow IHL, Albert DM. The color of the human eye: A review of morphologic correlates and of some conditions that affect iridial pigmentation. Surv Ophthalmol 1997;41:S117–S123
4. Eisinger M, Marko O. Selective proliferation of normal human melanocytes in vitro in the presence of phorbol ester and cholera toxin. Proc Natl Acad Sci USA 1982;79:2018–2022
5. Halaban R, Alfano FD. Selective elimination of fibroblasts from cultures of normal human melanocytes. In Vitro 1984;20:447–450
6. Gilchrest BA, Vrabel MA, Flynn E, Szabo G. Selective cultivation of human melanocytes from newborn and adult epidermis. J Invest Dermatol 1984;83:370–376
7. Halaban R, Ghosh S, Duray P, Kirkwood JM, Lerner AB. Human melanocytes cultured from nevi and melanomas. J Invest Dermatol 1986;87:95–101
8. Herlyn M, Mancianti ML, Jambrosic J, Bolen JB, Koprowski H. Regulatory factors that determine growth and phenotype of normal human melanocytes. Exp Cell Res 1988;179:322–331
9. Wilkins L, Gilchrest BA, Szabó G, Weinstein R, Maciag T. The stimulation of normal human melanocyte proliferation in vitro by melanocyte growth factor from bovine brain. J Cell Physiol 1985;122:350–361
10. Hu DN, McCormick SA, Ritch R, Pelton-Henrion K. Studies of human uveal melanocytes in vitro: Isolation, Purification and Cultivation of Human uveal melanocytes. Invest Ophthalmol Vis Sci 1993;33:2210–2219
11. Hu DN, McCormick SA, Ritch R. Studies of human uveal melanocytes in vitro: Growth regulation of cultured human uveal melanocytes. Invest Ophthalmol Vis Sci 1993;34:2220–2227
12. Hu DN, McCormick SA, Orlow SJ, Rosemblat S, Lin AY, Wo K. Melanogenesis in cultured human uveal melanocytes. Invest Ophthalmol Vis Sci 1995;36:931–938
13. Hu DN, McCormick SA, Orlow SJ, Rosemblat S, Lin AY. Regulation of melanogenesis by human uveal melanocytes in vitro. Exp Eye Res 1997;64:397–404

14. Hu DN, McCormick SA, Lin AY, Lin JY. TGF-β2 inhibits growth of uveal melanocytes at physiological concentrations. Exp Eye Res 1998;67:143–150
15. Hu DN, Stjernschantz J, McCormick SA. Effect of prostaglandin A_2, E_1, $F_{2\alpha}$ and latanoprost on cultured human iridal melanocytes. Exp Eye Res 2000;70:113–120
16. Lin JY, Hu DN, McCormick SA. Effects of neurotransmitters on human uveal melanocytes in vitro. Invest Ophthalmol Vis Sci 1997;38:S803
17. Zhao H, Li L, Boissy RE, Nordlund JJ. Ocular melanocytes express β-adrenergic receptor and respond to autonomic neurotransmitters. Pigment Cell Res 1997;10:119–120
18. Prota G. Regulatory mechanisms of melanogenesis: Beyond the tyrosinase concept. J Invest Dermatol 1993;125:297–303
19. Thody AJ, Graham A. Does α-MSH have a role in regulating skin pigmentation in humans. Pigment Cell Res 1998;11:265–274
20. Hunt G, Donatien PD, Lunec J, Todd C, Kyne S, Thody AJ. Cultured human melanocytes respond to MSH peptides and ACTH. Pigment Cell Res 1994;7:217–221
21. Abdel-Malek Z, Swope VB, Suzuki I, Akcali C, Hariger MD, Boyce ST, Urabe K, Hearing VJ. Mitogenic and melanogenic stimulation of normal human melanocytes by melanotrophic peptides. Proc Natl Acad Sci USA 1995;92: 1789–1793
22. Hedley SJ, Gawkrodger DJ, Weetman AP, MacNeil S. MSH and melanogenesis in normal human adult melanocytes. Pig Cell Res 1998;11:45–56
23. Boissy RE, Zhao H, Li L, Abdel-Malek Z, Suzuki I, Nordlund JJ. Ocular melanocytes are resilient to and do not express receptors for melanocyte-stimulating-hormone. Pigment Cell Res 1999;(Suppl. 7):39–40
24. Goodall T, Buffy JA, Rennie IG, Benson M, Parsons MA, Faulkner MK, MacNeil S. Effect of melanocyte stimulating hormone on human cultured choroidal melanocytes, uveal melanoma cells, and retinal epithelial cells. Invest Ophthalmol Vis Sci 1994;35:826–837
25. Haycock JW, Wagner M, Morandini R, Ghanem G, Rennie IG, MacNeil S. Alpha-melanocyte-stimulating hormone inhibits NF-kappaB activation in human melanocytes and melanoma cells. J Invest Dermatol 1999;113:560–566
26. Roberts JE, Wiechmann AF, Hu DN. Melatonin receptors in human uveal melanocytes and melanoma cells. J Pineal Res 2000;28:165–171
27. Coleman RA, Smith WL, Narumiya S. Classification of prostanoid receptors: Properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 1994;46:205–229
28. Selen G, Stjernschantz J, Resul B. Prostaglandin-induced iridial pigmentation in primates. Surv Ophthalmol 1997;41:S125–S128
29. Wistrand PJ, Stjernschantz J, Olsson K. The incidence and time-course of latanoprost-induced iridal pigmentation as a function of eye color. Surv Ophthalmol 1997;41:S129–138
30. Lake S, Krook K, Ludgren M, Arvidson M, Hu DN, Stjernschantz J. Effect of latanoprost on the transcription of tyrosinase and TRP1 in cultured human iridial melanocytes. Invest Ophthalmol Vis Sci 1997;38:S814
31. Abdel-Malek Z, Scott MC, Suzuki I, Tada A, Im S, Lamoreux L, Barch G, Hearing V. The melanocortin-1 receptor is a key regulator of human cutaneous pigmentation. Pigment Cell Res 1999;(Suppl. 7):44
32. Berman ER. Biochemistry of the Eye. New York: Plenum Press; 1991. pp. 151–165
33. Prota G, Hu DN, Vincensi MR, McCormick SA, Napolitano A. Characterization of melanins in human irides and cultured uveal melanocytes from eyes of different colors. Exp Eye Res 1998;67:293–299
34. Marmor MF, Wolfensberger TJ. The retinal pigment epithelium: Function and Disease. New York: Oxford University Press; 1998. pp. 103–172
35. Hu DN, McCormick SA, Lin Y, Ritch R. Comparison of cultured iris and retinal pigment epithelial cells: Production and response to growth factors. Invest Ophthalmol Vis Sci 1998;39:S580

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The Melanocyte

Its Structure, Function, and Subpopulations in Skin, Eyes, and Hair

*Raymond E. Boissy, Ph.D.**

The primary function of melanization is to synthesize an optically dense pigment polymer to shield other cell types from the damaging effect of the sun. Sometimes, this melanin polymer, in the form of an organelle called the melanosome, is transferred to neighboring cells, whereas in other situations, it is kept by the melanocyte itself. Pigment synthesis may not be the sole function of the pigment cell, as current research is indicating that the melanocyte may be involved in regulating other biologic processes. These other possible functions will be discussed by other authors in this volume.

In this article, I review the basic structural and functional correlates of the melanocyte, addressing the embryonic establishment of the melanocyte, the synthesis and translocation of melanin, and the eventual transfer of pigment to epithelial cells. There are dramatic structural and functional differences within the melanocyte population, and these differences can be used to classify melanocytes into three subtypes that are restricted to specific anatomic sites: the cutaneous epidermis, the uveal tract, and the hair. While reviewing the general process of melanization, I have frequently alluded to the differences that exist between the three subtypes of pigment cells.

ESTABLISHMENT OF MELANOCYTES

Melanocytes are embryonically derived from a stem population of melanoblasts that originate from neural crest cells soon after closure of the neural tube.[66, 82] Emigration of melanoblasts from this embryonic source begins as early as 2½ weeks of gestation in human embryos[5] and at 3½ days in avian embryos.[42, 82] These early neural crest cells are committed to differentiate into melanocytes either before or during their migration out of the neural tube. This has been demonstrated by the success in developing pure cultures of melanocytes from mechanically and enzymatically isolated neural tubes.[7, 44] The first cells that migrate out of the neural tube onto the culture substratum are stellate and produce pigment that is apparent microscopically after 6 days. However, in vivo, the melanoblasts of most species begin melanization just before or just after they have reached their destination.[56] In monkeys, this occurs for the cutaneous melanocyte at about week 10 in fetal development and for the uveal melanocytes at week 20.[27] It appears that both of these subtypes of melanocytes migrate out of the entire posterior two thirds of the neural tube,[6] suggesting that there is a postemigration control point that influences the development of one subtype of melanocyte, prevents the differentiation of the other type, or both. The observation that uvea-like melanocytes, which contain larger granules than cutaneous melanocytes, originate from more caudal areas of the neural tube can be explained in another way. These extracephalic melanoblasts may be precursors of dermal melanocytes, which, in lower vertebrates, morphologically resemble uveal melanocytes.[2] Dermal melanocytes or melanophores contain relatively large

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melanosomes and have not been shown to synthesize additional pigment granules once they have been established prenatally.

Although dermal melanocytes are not routinely present in higher vertebrates, there are a few instances where they have been documented. For example, dermal melanocytes exist in the ears, soles, tail, scrotum, muzzle, and genital papilla of rodents[3, 64] and are prominent throughout the PET mouse,[67] where they appear to contain relatively large melanosomes.[68] In humans, continent dermal melanocytes filled with melanosomes up to 1 μm in diameter can occur congenitally in the nevus of Ota,[41] the blue nevi,[28] and the Mongolian spot.[38] This speculation about the potential existence of dermal melanocytes in mammals and humans strengthens the hypothesis that a post-neural-crest emigration control point influences the development or suppression of various subpopulations of melanocytes during embryogenesis. Once the melanocytes have established themselves in their target organ, that is, skin, hair, or eyes, they remain there for the life of the individual. The hair melanocytes may be a possible exception to this rule that will be addressed later in this article.

At birth, melanocytes are well established in the epidermis and transfer melanosomes to keratinocytes.[18] These melanocytes remain in the basal layer of the epidermis, only rarely dividing[31] or migrating away. The quantity of melanocytes per area of skin at various body sites in the human varies dramatically (about three-fold); however, the whole-body average ranges from 800 to 1600/mm^2.[17] Some of the more congested areas are the sun-exposed skin and, ironically, the least sun-exposed areas, that is, the genitals. The quantity of melanocytes per area decreases with age.[17]

Uveal melanocytes are also well established in the choroid, ciliary body, and iris at birth. Pigment synthesis in these melanocytes begins during late gestation, 140 days, and by birth, the uveal melanocytes have almost completed their quota of melanosomes. However, the uveal melanocytes may still be melanogenically active at birth. Choroids in chickens at day of hatch demonstrate electron-dense reaction products in part of the Golgi apparatus and in vesicles radiating from it after the tissue has been treated by dopa histochemistry for the localization of tyrosinase (personal observation). In addition, a few stage one premelanosomes exist in the perikaryon of uveal melanocytes in this early postnatal period. In short order, however, these active uveal melanocytes will cease melanization altogether and remain melanogenically dormant throughout life.[12, 25, 45]

The cutaneous melanocytes differ dramatically from the uveal melanocyte in the duration of time they are involved in melanization. Melanocytes of the epidermis are continuously synthesizing melanosomes and transferring them to keratinocytes throughout life. These melanocytes are found exclusively in the basal layer of the epidermis; in fact, the ventral side of the perikaryon frequently appears to dip down into the dermis pendulously while still resting on a basement membrane. The dendrites of these basal melanocytes reach up intercellularly through the epidermis to the stratum spinosum. The melanocyte is loosely anchored in this position. Forms of intercellular contact with keratinocytes by desmosomes and gap junctions, or attachment to the basement membrane by hemidesmosomes, do not exist in the melanocyte. Also of interest is the absence of dermal anchoring fibrils beneath the melanocytes proper (G. Moellmann; personal communication). Anchoring fibrils, produced by dermal fibroblasts, are selectively placed beneath basal epidermal cells, where they connect the lamina densa of the basement membrane with the collagen fibers deeper in the dermis. In all other aspects, however, the basement membrane beneath the melanocyte appears morphologically normal. Since the melanocyte is not riveted into the epidermal complex, one wonders whether it can be mobile. However, no evidence exists clearly demonstrating that the melanocytes migrate around in the epidermis or that their dendrites dynamically weave about between the suprabasal keratinocytes.

Uveal melanocytes are also relatively immobile in the stroma of the choroid, the iris, and the ciliary body. These fully differentiated and metabolically dormant uveal melanocytes contain numerous melanosomes that fill up the cytoplasm in such a congested manner that nuclear indentation occasionally results from the pressure of the granules (Fig. 1). The melanosomes synthesized by the uveal melanocyte are predominantly spherical, homogenously pigmented, and large (approximately 0.8 μm in diameter). These differ from the smaller granules produced by the cutaneous melanocytes as described below. Interestingly, the melanocytes in the leptomeninges of the human brain,[19] also of neural crest origin, mimic the structural and dormancy characteristics of the uveal melanocytes. The uveal melanocytes also contain only a minimal amount of endoplasmic reticulum, Golgi apparatus, and mitochondria,

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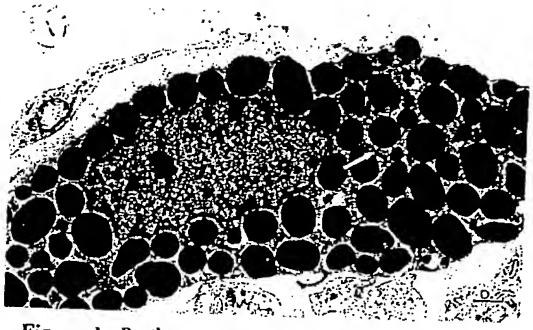

Figure 1. Perikaryon of choroidal melanocyte from an adult (1-year-old) chicken. Melanosomes are heavily pigmented and spherical to oblong and have an average diameter of 0.8 μm. The cytoplasm is congested with melanosomes and has very few other components. There is some rough endoplasmic reticulum *(arrow)*. Scale bar = 0.67 μm.

suggesting that these cells on the whole are relatively inactive, generating only a small amount of those products needed exclusively for maintenance. The melanosomes remain within the uveal melanocyte and are never transferred out of the cell. However, they do demonstrate subtle morphologic changes with age.[26]

MELANOSOMAL TRANSFER

Transfer of melanosomes to a neighboring cell is one of the main functions of the epidermal melanocyte. It has been estimated that 36 keratinocytes are serviced by a single basal melanocyte.[16] This servicing consists of the transfer of the melanocyte's luxury product, the melanosome, into epithelial cells advancing up through the epidermis as they differentiate into keratinocytes. At times, these transferred melanosomes form a cap over the dorsal face of the nucleus in the keratinocyte. It has been advocated for years that the function of the pigmented dendrites of the melanocyte, as well as of the melanosomes transferred into keratinocytes, is to shield and protect the genome of the proliferating basal epithelial cells from the damaging effect of ultraviolet light. Recently, it has been suggested that the pigment in both the melanocytes and the keratinocytes also acts as a scavenger of photogenerated free radicals, again preventing cellular DNA damage.[57]

In any case, melanosomal relay from the tip of the melanocytic dendrite to the keratinocyte is a process in which slight variations exist between ethnic groups. There are only two aspects of the melanization process that differ among persons of different racial skin color: the size of melanosomes and the manner in which they are distributed within the recipient keratinocyte. In contrast, the number and morphology of the melanocytes as well as their tyrosinase activity and the number of melanosomes they produce are fairly constant among all ethnic groups.[75] There is, however, one summary report that suggests that the total number of melanosomes per given area increases from the Caucasoid to the Mongoloid and to the Negroid skin.[34] Most of the data in the literature attest that in Caucasoid skin, melanosomes are smaller, averaging less than 0.8 μm,[78] and are distributed within keratinocytes as membrane-bound complexes containing approximately one-half dozen granules plus small particulate debris.[76] In Negroid skin, melanosomes are slightly larger (greater than 0.8 μm) and dispersed individually throughout the keratinocyte. There is a variation in melanosomal size, not only between ethnic groups but also within an ethnic group. In Negroid keratinocytes, the relative size of the melanosomes transferred to keratinocytes correlates with the density of the skin tone; the larger the melanosome transferred, the darker the resulting complexion.[79] The more dispersed and larger granules will absorb light more efficiently and thus give the skin its darker color.

It appears that the size of the melanosome synthesized by the basal melanocyte governs the manner in which it is transferred to the keratinocyte. The most convincing data are derived from the circumscribed pigmented macules that appear in patients treated chronically with photochemotherapy (PUVA).[69] In these lesions, the melanocytes become relatively hypertrophic and sometimes cytologically atypical. Interesting ultrastructural observations were reported regarding melanosome size and distribution pattern in keratinocytes in the lesion versus the adjacent sun-protected normal skin.[55] In the basal keratinocyte, the melanosomes are significantly larger in the lesion than in the unaffected area. Correlating with this difference is the prominent appearance of single melanosomes in the lesional keratinocyte as opposed to the numerous compound melanosome complexes in the normal keratinocyte. Other researchers have shown that the size of the melanosome determines its distribution pattern in the keratinocyte.[78] That is, there is a tendency for small melanosomes (less than 0.24 μm) to be complexed and for larger ones to be dispersed individually in basal keratinocytes in some states of hyperpigmentation in Caucasoid

skin.[39] In contrast, Seiji and coworkers[72] have stated that the keratinocyte rather than the melanocyte controls the morphologic fate of transferred melanosomes. These investigators have observed that large phagocytosed compartments enter all keratinocytes and subsequently get dispersed singly or as smaller complexes with a few melanosomes, depending on the ethnic group. This suggests that the keratinocyte controls the distribution of its incorporated melanosomes.[72]

It is still unclear whether the melanocyte, the keratinocyte, or both is the active partner in the process of melanosome transfer. Review of the literature suggests that the two cell types are equally involved. In co-cultures of epithelial cells and melanocytes, it is the latter cell that migrates about. The "active" melanocyte will also extend its dendrite up against the plasma membrane of the epithelial cell, forming a large vacuole that eventually pinches off into cytoplasm.[72] On the other hand, when isolated keratinocytes are co-cultured with latex beads, the latter readily get phagocytized by the "aggressive" keratinocyte.[84] In addition, cultured guinea pig keratinocytes, when incubated in medium containing isolated melanosomes, will take up small melanosomes extracted from B16 mouse melanomas as small complexes and conversely will take up large melanosomes extracted from B16 mouse hair bulbs singly.[83] Current work is being done on the skin equivalent model designed by Eugene Bell.[80] In this system, human neonatal melanocytes are seeded onto a dermal equivalent before it is overgrown by keratinocytes. When this elaborate culture system is irradiated with ultraviolet B, pigment granules transferred into neighboring keratinocytes are compartmentalized as small clusters. It is hoped that future experimental manipulations on this system will reveal the complete mechanism of pigment transfer. However, there is another very intriguing observation that was reported by those investigators.[80] In the development of their skin equivalent model, some melanocytes were trapped in the dermal component of the system. These melanocytes were heavily pigmented and congested with a large number of melanosomes, some being extremely perinuclear. The authors speculated that these isolated cells have no apparent relation with keratinocytes and therefore no neighboring target that stimulates granule discharge. If one were to speculate from this result, I would suggest that the melanocytes in the uveal tract and the leptomeninges appear dormant because they lack the partner that is needed to perpetuate the transfer of their granules.

MELANOSOME TRANSLOCATION

Immediately prior to the transfer of melanosomes to the keratinocyte, the melanocyte must translocate its melanosomes, synthesized within its perikaryon, down the dendrites to the site of transfer. Intermediate filaments 9 to 10 nm in diameter are used to translocate the melanosomes out through the dendrites. These filaments have been called intermediate filaments because their diameter falls between that of actin-like filaments (5 to 7 nm) and microtubules (25 nm). The role of these intermediate filaments has been most comprehensively studied in association with inducers of pigmentation, namely melanocyte-stimulating hormone (MSH) and ultraviolet B. Immediate tanning (darkening of the skin within minutes and up to 3 hours after exposure to ultraviolet light) results in hypertrophy of the epidermal melanocyte: an elongation of the dendrites plus an increase in the number of melanosomes transferred to keratinocytes. Ultrastructurally, this event corresponds to a shift of 10-nm filaments from the perinuclear area to the medial areas throughout the dendrites. The filaments are also closely juxtaposed to the melanosomes, suggesting that these 10-nm filaments in human melanocytes participate in the elongation of the dendrites and the translocation of melanosomes. Microtubules, on the other hand, are seen mingled with the 10-nm filaments in the perikaryon prior to ultraviolet B exposure. However, during ultraviolet B–induced dendritic elongation, microtubule reorientation is restricted to the underside of the plasma membrane, suggesting that they are responsible for the dendrite elongation and not directly for melanosomal translocation.[31]

The role of microtubules and 10-nm intermediate filaments has also been studied extensively in dermal melanocytes of amphibians[47, 53] and of the teleost fish,[48] with contradictory results. *Rana pipiens* rapidly darkens as a result of melanosomal dispersion down dendrites by various inducers such as MSH, theophylline, and dibutyryl cAMP. This process can be either inhibited or reversed after treatment with cytochalasin B, a mold metabolite that disrupts microfilaments. After exposure to cytochalasin B, amphibian melanocytes lighten, and their melanin granules retract into the perikaryon. Microfilaments are no longer apparent ultra-

structurally, and thus granule dispersion cannot be maintained. Conversely, the process of dispersion is not affected by the microtubule inactivators such as colchicine and vinblastine, which are plant alkaloids that prevent microtubule subunit assembly or precipitate tubulin as large crystals, respectively. On the other hand, the aggregation of melanosomes back into the perikaryon is controlled by microtubules in this system and can be prevented by pretreatment with microtubule disruptors and thus reverse translocation. On the other hand, McNiven and Porter[48] recently demonstrated that microtubules are responsible for translocation of pigment granules both up and down the dendrites in melanophores cultured from the teleost fish. In this system, the polarity of the microtubules confers direction to granule translocation. These differences in the control of translocation may be attributed to species differences that could have evolved as a result of the speed of color alteration needed in each species.

Interestingly, a current report has suggested that the immediate pigment darkening in human skin by ultraviolet A exposure is a passive, acellular photochemical reaction.[24] Pigment darkening still occurs in the test system even at 0°C and after fixation, filament and microtubule destruction, and continuous freezing and thawing. Ironically, the usual fading of the immediate pigment darkening does not occur in any of the above experimental systems. This suggests that only aggregation is an active cellular process.

PIGMENT SYNTHESIS WITHIN THE MELANOCYTE

The synthesis of melanosomes for transfer to keratinocytes is a bipartite process. The two major components manufactured by the melanocytes are the premelanosomal organelle and the tyrosinase enzyme. These two components can interact to produce two types of pigment granules: the brown/black eumelanosome and the yellow/red pheomelanosome. The former granule is an oval organelle approximately 0.9 μm in length and 0.3 μm in diameter, whereas the latter granule is more spherical, with a diameter of 0.7 μm. In most situations, the melanocyte will synthesize one of the two types of melanosomes exclusively; however, in the agouti mouse,[71] the same melanocyte can switch its synthesis from one type of granule to the other during a cycle of hair regeneration.

Both types of premelanosomes have an internal matrix of melanofilaments. These filaments are arranged in an orderly hexagonal pattern in eumelanosomes (Fig. 2) but are very disorganized and irregular in the pheomelanosome. The matrix is predominantly of glycoprotein, with striking molecular differences between the two granular types, possibly accounting for their structural differences.[37] The premelanosome originates as a dilation of the smooth endoplasmic reticulum (Fig. 2). Some investigators have suggested that this reticulum is an immediate extension of part of the Golgi apparatus[46] or, more specifically, part of the Golgi-associated system of smooth endoplasmic reticulum (GERL).[58]

The tyrosinase enzyme also travels through the Golgi apparatus. Its polypeptide core is translated by rough endoplasmic reticulum (RER)-bound ribosomes and then shuttled through the Golgi apparatus for glycosylation. The more mature enzyme then leaves the trans face of the Golgi apparatus in coated vesicles[51] or tubules[13] and migrates to neighboring premelanosomes in the perikaryon (Fig. 2). After fusion of the coated vesicle with the premelanosome, melanin synthesis begins. This process starts with the dihydroxylation of tyrosine to dihydroxyphenylalanine (L-dopa), which is then converted to dopa quinone.[43] Both reactions are catalyzed by tyrosinase. The first step is presumed to be rate limiting. Once dopa accumulates, it works as a cofactor to accelerate the first reaction.

It has been assumed for years that these two reactions were the only enzymatically controlled steps in melanization. However, recent work has identified at least three other control points in this process, functionally named dopachrome conversion factor, indole blocking factor, and indole conversion factor, the last possibly being another function of the tyrosinase molecule.[40, 61] During pheomelanogenesis, cysteine combines with dopa quinone nonenzymatically to yield cysteinyldopa, which is then oxidized and incorporated into pheomelanin.[63] As melanization occurs within either type of premelanosome, the melanin polymer is deposited on the melanofilament in an additive fashion until the entire organelle becomes homogenously pigmented (Fig. 3). During the initial stages of the premelanosomes, vesiculoglobular bodies with a diameter of 4 nm can be observed in the granule (Fig. 2), predominantly along its periphery. The nature of these subunits is still unknown; however, it has been suggested that they are the tyrosinase-carrying coated vesicles that fuse with the premelanosome and invert

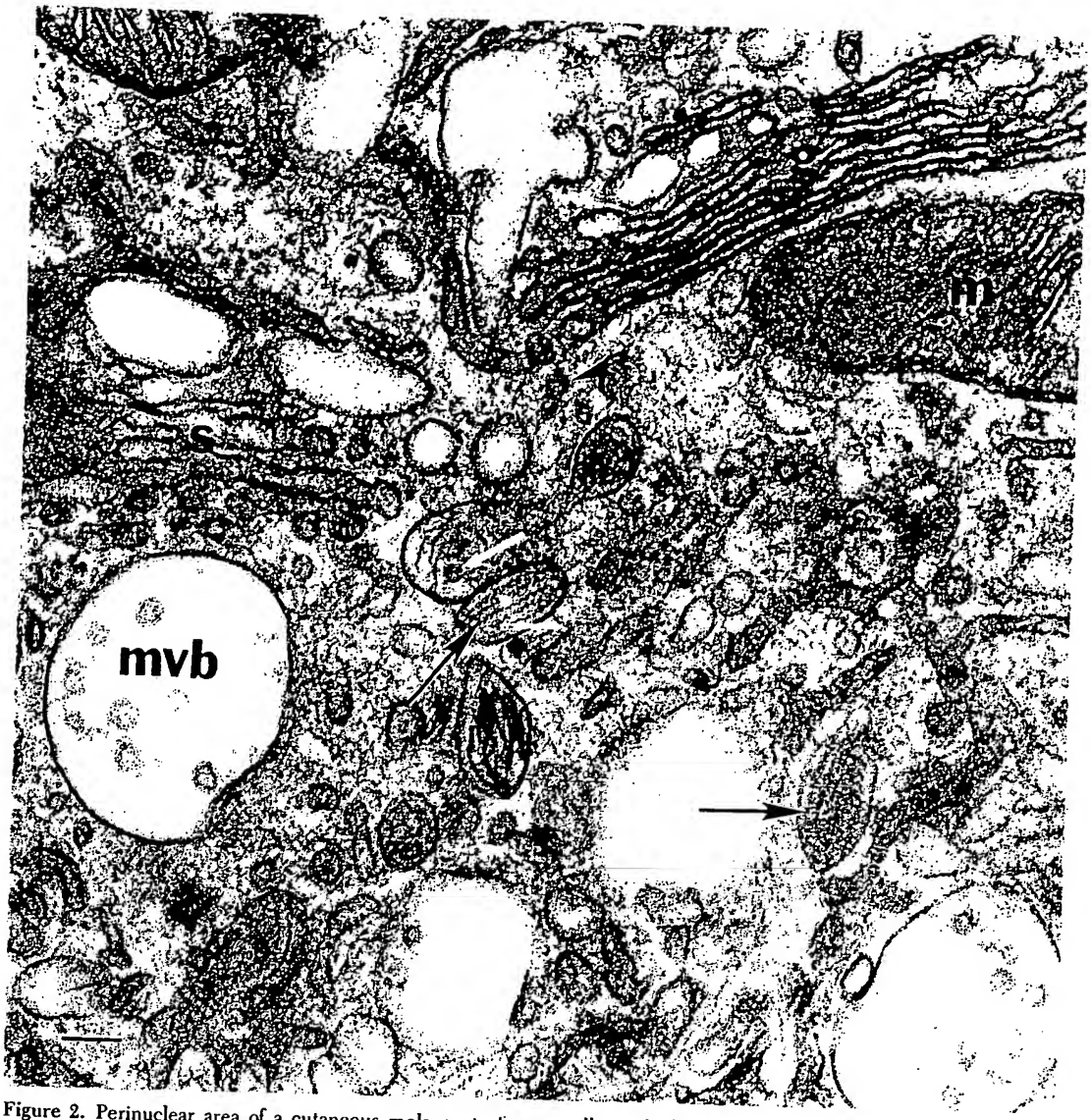

Figure 2. Perinuclear area of a cutaneous melanocyte from an albino chicken. Many of the structural components of melanization are apparent: Golgi apparatus (g), from which bud coated vesicles *(arrowhead)* carrying tyrosinase; smooth endoplasmic reticulum (s), from which originate the premelanosomes, with their matrix of melanofilaments *(arrows)*; and vesiculoglobular bodies *(white arrow)* (mvb = multivesicular body; m = mitochondria). Scale bar = 0.12 μm.

during incorporation and delivery of the enzyme.[32, 81] Incubation of melanocytes with L-dopa is often used histochemically to stain sites of functional tyrosinase activity.[62] Electron-dense reaction product, presumably induced melanin, is localized on one or two trans-most cisternae of the Golgi apparatus, in vesicles and tubules radiating from it, and in some but not all of the neighboring premelanosomes (Fig. 3). This suggests that tyrosinase is functional even before it leaves the Golgi apparatus and is completely glycosylated. Therefore, in the de novo condition, the substrate is not encountered by tyrosinase until the enzyme is incorporated into premelanosomes, or the enzyme is held in a nonfunctional state by a system not resistant to histochemical processing.

Many isozymes of tyrosinase have been identified in melanoma cells[22, 59] as well as in normal melanocytes.[14, 20] These isozymes, as resolved by gel electrophoresis, range in molecular weight from 65 to 81.2 kD.[22] The largest form of tyrosinase is an insoluble molecule that is tightly complexed within the melanosome and presumed to be the final functional form of the enzyme.[23] The smaller forms are all precursors

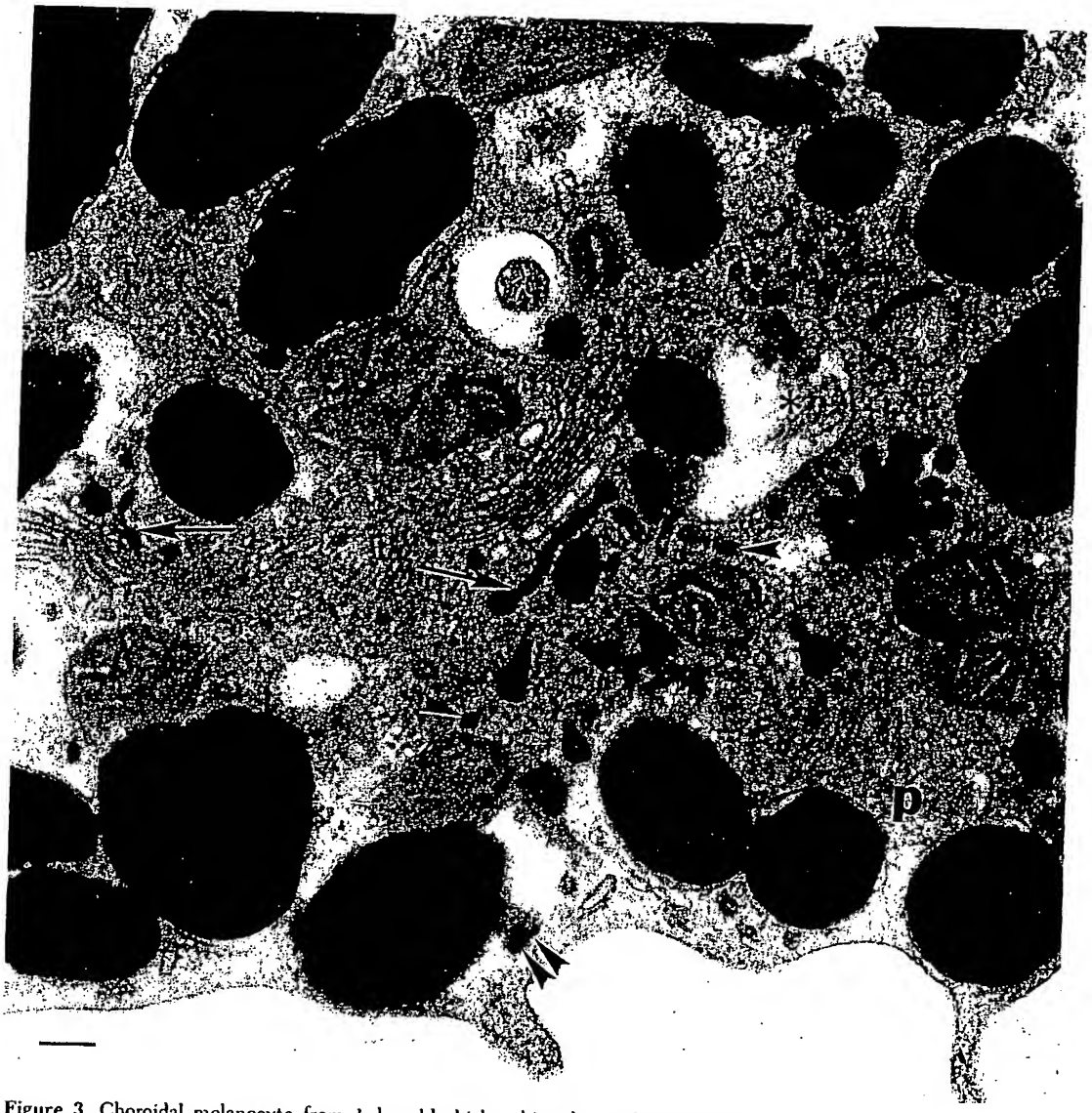

Figure 3. Choroidal melanocyte from 1-day-old chicken histochemically stained for tyrosinase localization by L-dopa incubation. Dopa-positive reaction product exists in the trans cisternae of Golgi apparatus *(arrows)* and in vesicles or tubules budding off the Golgi apparatus and migrating to the melanosomes *(arrowheads)*, which eventually fuse with pigment granules *(double arrowhead)*. Note melanofilaments forming in a dilated area of the smooth endoplasmic reticulum *(asterisk)* (p = premelanosome). Scale bar = 0.18 μm.

with various amounts of sugar and sialic acid residues, which are added sequentially to the core polypeptide as it travels through the Golgi apparatus. As mentioned in the previous paragraph, these less-glycosylated intermediates are enzymatically functional. All bands of tyrosinases isolated from cell extracts onto SDS gels will convert L-dopa to melanin when stained histochemically with the intermediate substrate. Also, when melanocytes are fixed, incubated with dopa, and processed for ultrastructural histochemistry, reaction product is identified in the Golgi apparatus (Fig. 3). Therefore, complete glycosylation of tyrosinase is not necessary for the activation of this enzyme.

It appears, however, that glycosylation of the tyrosinase polypeptide is essential for its translocation through the Golgi apparatus to the premelanosome. Glycosylation of tyrosinase can be inhibited by both glucosamine and tunicamycin. Such treatment results in the absence of the higher-molecular-weight forms of tyrosinase[29] and prevents the nascent protein from exiting the Golgi apparatus.[30] Such glyco-

sylation inhibitors also interfere with the assembly of melanofilaments in the premelanosome,[30] verifying that these structures are glycoproteins. That the translocational signal is a property of sugar residues on enzymes has been demonstrated in the lysosomal system.[9, 21] Mannose residues on lysosomal enzymes are phosphorylated in the Golgi apparatus and then bound to a specific membrane receptor as it buds off into coated vesicles. The receptor-enzyme complex migrates selectively to an endosome or lysosome, where the enzyme is deposited and the receptor recycles back to the Golgi apparatus to translocate another lysozyme molecule.[9] A receptor for tyrosinase responsible for its translocation to the premelanosome has yet to be identified. However, the possibility exists. Tyrosinase has a large complex of asparagine-linked sugar chains that include a high-mannose type.[59] These mannose residues, or other portions of the carbohydrate side chains, could denote a signal for the translocation of tyrosinase. An alternative or collateral mechanism for translocation could be acidification. More recent evidence shows that the movement of membrane organelles in the endocytotic and exocytotic pathway may be regulated by their relative internal pH.[49] More specifically, the trans face of the Golgi apparatus and forming secretory vesicles in human fibroblasts are acidic and have been postulated to be involved in the translocation of fibronectin in human fibroblasts.[1] Could this process be extended to the melanocyte system, where tyrosinase translocation from the Golgi apparatus, as well as its activation within the premelanosome, is coordinated by pH gradients? It has already been suggested that the premelanosome or translocating vesicles or both are acidic vesicles in which tyrosinase activity is suppressed until some unidentified process normalizes the pH of these compartments. Treating cultures of mouse melanoma cells with ionophores that collapse the transmembrane pH gradient, such as monensin or nigericin, increases tyrosinase activity.[70] It is apparent from all these questions that the search for the intracellular mechanisms controlling melanin synthesis is one of the frontiers in pigment cell research.

MELANOCYTES OF THE HAIR

The melanocytes in regenerating hair can be considered a separate population of cells from the cutaneous and uveal populations because they synthesize melanin intermittently. Unlike the continuous melanization and transfer of melanosomes by the cutaneous melanocyte and the postnatal dormancy and melanosome retention of the uveal melanocyte, the hair melanocyte produces pigment cyclically, during each period of active hair growth (as addressed below). On the other hand, hair melanization is functionally similar to skin melanization in that these melanocytes produce pigment for transfer to the epithelial cells of that particular organ, namely skin and hair. The coloration of that organ is subsequently determined by the status of the transferred pigment (see article by Dr. Cline).

The embryonic development and the architecture of a proliferating hair is a slight modification of that seen in the skin. During development of the hair shaft, the epithelial germ, originating from basal ectoderm, grows down into the dermis at 3 months of gestation. As the hair germ cells elongate and form a hair shaft, encasing basally a dermal papilla, the dopa-positive melanocytes of the skin epidermis comigrate and are randomly distributed in this forming structure. Not until 6 months of gestation, when the eventual complete bulbous peg stage is formed, do the melanocytes preferentially exist in the outermost epithelial layer immediately capping the dermal papillae.[52] As in the skin, hair melanocytes end up on the basement membrane, unanchored by desmosomes and tonofilaments, and extend dendrites toward the cortical cells (Fig. 4*A*) to which melanosomes are transferred as the cortical cells migrate out of the germinal layer to the hair shaft proper.[54] However, in the basal epithelial layer, there is a one-to-one ratio of melanocytes to hair epithelial cells as opposed to the predominance of basal epithelial cells over melanocytes ocurring in the skin.

Melanosomal transfer appears to resemble initially the process occurring in Caucasoid skin in which packets of granules are transferred. Mottaz and Zelickson[54] have demonstrated that portions of dendritic processes are phagocytized by the cortical cells of the hair. These internalized granules stay in these membrane-bound complexes for a very short time until the membrane breaks down and the melanosomes get dispersed throughout the cortical cell (Fig. 4*B*).[54]

The bipartite system of melanin synthesis described earlier for cutaneous and ocular melanocytes also takes place in the hair melanocyte (Fig. 4*C*).[36] However, there is one ultrastructural difference. In the hair, the morphologic difference between the eumelanosomes of

Figure 4. Melanization of hair bulbs during the anagen growth stage in a C57BL mouse. *A*, Light micrograph of regenerating hair bulb; mitotic epithelial cells *(arrow)* proliferate at the base of the bulb, migrate up the shaft, and receive pigment from melanocytes *(arrowhead)*. Note cortical cells in which the transferred pigment caps the apical surface of the nuclei *(asterisk)*. Scale bar = 40 μm. *B*, Low-power electron micrograph of hair bulb with numerous active melanocytes (m) residing on the dermal papilla/epidermal junction *(arrowhead)* and neighboring epithelial cortical cells (e), which have received melanosomes. Note that transferred melanosomes are distributed singly and in membrane-bound complexes *(arrow)* within epithelial cells (dp = dermal papilla). Scale bar = 2.44 μm.

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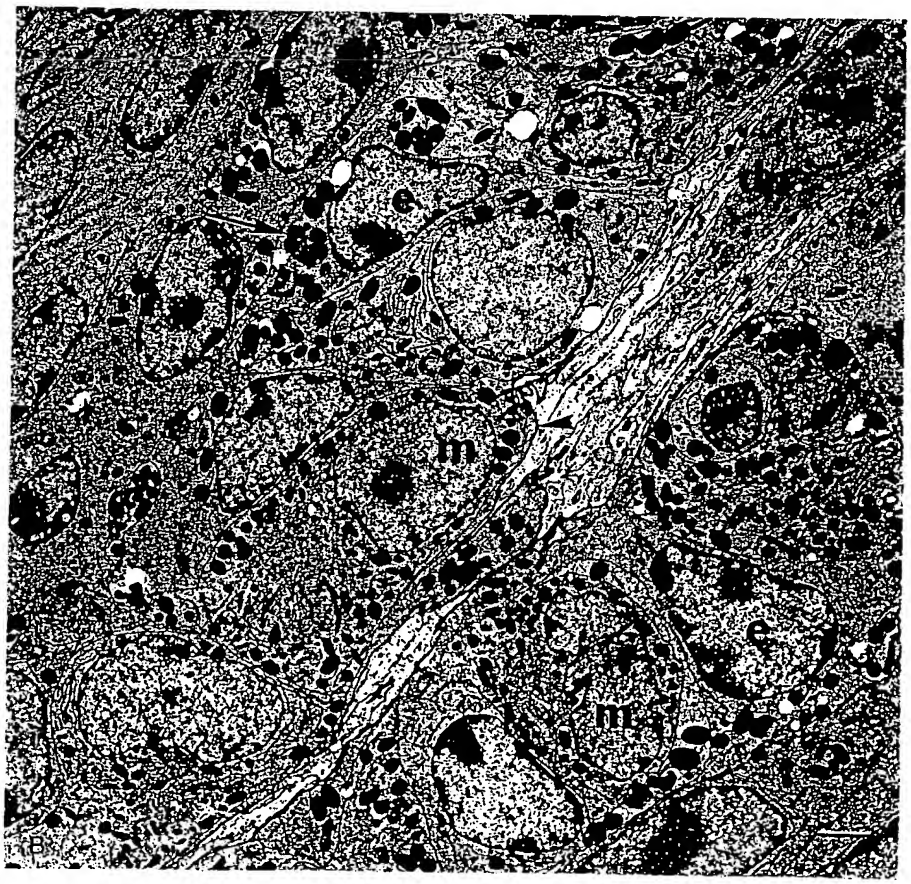

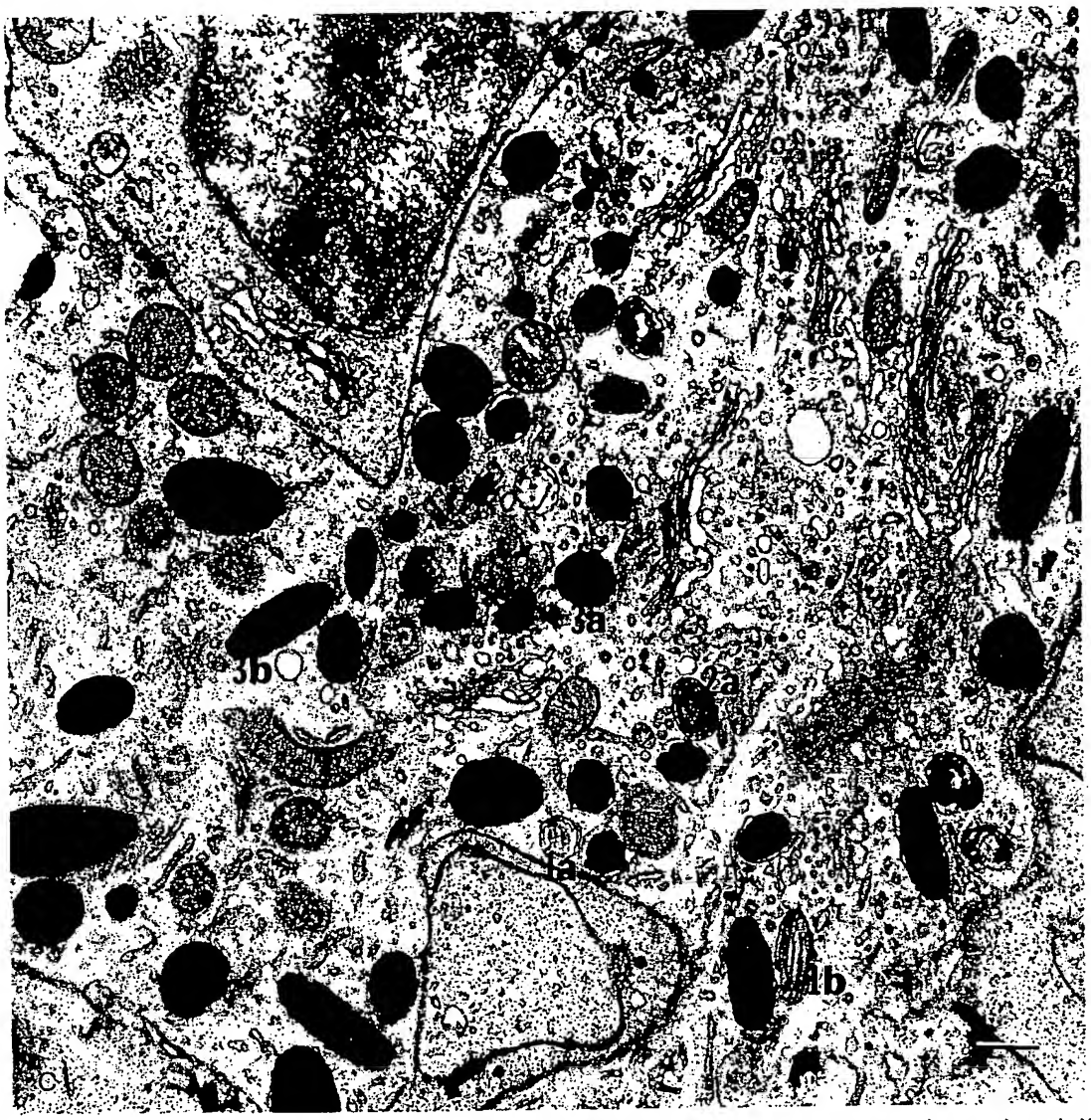

Figure 4. *Continued.* C, High-power electron micrograph of the perikaryon in a hair melanocyte. This area has a half-dozen stacks of Golgi apparatus and numerous melanosomes in various stages of melanization; that is, premelanosomes (1) and partially melanized (2) and completely melanized (3) eumelanosomes in cross (a) and longitudinal (b) section. Scale bar = 0.27 μm.

black/brown/blond hair and the pheomelanosomes of red hair is not clearly distinguishable in hair from mice as they are in either the skin[11, 33] or feathers.[10] Eumelanosomes produced by the hair melanocytes are indeed ellipsoidal in conformation, and the extent of melanin deposition on the melanofilaments correlates with hair color; that is, deposition decreases from black to brown to blond. In addition, the initial pheomelanosome synthesized by the melanocyte is ellipsoidal like the eumelanosome.[77] It is not until its transfer into the cortical cell that it conforms to the typical pheomelanosome shape, which is spherical, with irregular and partially pigmented melanofilaments.[4]

Hair growth is a cyclic process that has been categorized into three stages: the anagen or proliferation stage, the catagen or termination stage, and the telogen or quiescent stage. In the scalp, the first two stages combined last 1000 days, whereas the resting telogen stage lasts 100 days.[60] Hair melanocytes synthesize pigment only during the anagen stage of growth. It has not been definitively demonstrated what happens to these melanocytes during the autolytic involutionary events of catagen

and during the telogen stage. In fact, during the telogen stage, the dormant hair is secured immediately below the epidermal-dermal junction, and the dermal papilla and remnants of the epithelium from the hair retract from the hair shaft and reside in the deeper area of the dermis. During the next cycle of hair regeneration, these few remaining epithelial cells proliferate and form a new hair bulb. It has been proposed that the melanocytes stop pigment production at the onset of catagen, and that some or all of the melanocytes remain in the epithelial germ cap as it retracts with the dermal papilla during telogen. Upon the subsequent round of hair growth, the melanoblast reactivates or proliferates to supply the pigmentation for the next hair.

Electron microscopic analysis of this process has demonstrated that melanocytes, identified as cells with a few small premelanosomes, do exist in the epithelial collar during catagen and in the hair germs during telogen.[74] It has been suggested that these melanocytes dedifferentiate during telogen, are reactivated in the subsequent anagen, and keep recycling until they are shed with a hair shaft prior to graying.[73] An alternative would be the existence of a reserve pool of neural crest–derived melanoblasts from which new melanocytes are provided with each hair growth cycle while the previous melanocyte populations are shed with the hair. This process is presumed to occur during the pigmentation of avian feathers.[15] In these animals, an inconspicuous population of melanoblasts resides in the dermis immediately outside the feather follicle. As each round of feather synthesis occurs, a portion of these stem melanoblasts migrates to the feather epithelium,[65] proliferates, synthesizes melanosomes, and transfers granules to feather epithelial cells. At the end of feather regeneration, the melanocytes become incorporated into the keratinized structure[8] and are shed with the feather during its molt. Graying or vitiligo-like amelanosis or both results when these melanoblasts are exhausted, prevented from migrating, or immunologically eliminated. Whether such a system is present in mammalian hair has yet to be shown.

SUMMARY

I would like to stress that there seem to be three subpopulations of neural crest–derived melanocytes in the body that can be functionally and morphologically distinguished: the cutaneous melanocytes, which continuously synthesize small melanosomes to be transferred to keratinocytes; the uveal melanocytes, which synthesize larger melanosomes for only a short while to be retained by this melanogenically dormant cell; and the hair melanocyte, which intermittently produces melanin either in a cyclic manner or as a periodic supply from a stem population. These three types of melanocytes synthesize melanin granules by an identical bipartite system. However, the control mechanism regulating the specific differentiation and postmelanin synthesis function of these cell types needs to be addressed in future research.

REFERENCES

1. Anderson RGW, Pathak RK: Vesicles and cisternae in the trans Golgi apparatus of human fibroblasts are acidic components. Cell 40:635–643, 1985
2. Bagnara JT, Matsumoto J, Ferris W, et al: Common origin of pigment cells. Science 203:410–415, 1979
3. Billingham RE, Silvers WK: The melanocytes of mammals. Q Rev Biol 35:1–40, 1960
4. Birbeck MSC, Barnicot NA: Electron microscope studies on pigment formation in human hair follicles. In Gordon M (ed): Pigment Cell Biology. New York, Academic Press, 1959, pp 549–561
5. Bolanole RP: The neurocristopathies: a unifying concept of disease arising in neural crest maldevelopment. Hum Pathol 5:409–429, 1974
6. Boissy RE: Identifying cutaneous and uveal melanocytes in cultures from avian neural tubes. J Invest Dermatol 87:386, 1986
7. Boissy RE, Halaban R: Establishment of proliferative, pure cultures of pigmented chicken melanocytes from neural tubes. J Invest Dermatol 84:158–161, 1985
8. Bowers RR, Chun DW: Tyrosinase and acid phosphatase pathways in regenerating feather melanocytes of the fowl. In Seiji M (ed): Pigment Cell 1981: Proceedings of the 11th International Pigment Cell Conference, Sendai, Japan, 1980. Tokyo University of Tokyo Press, 1981, pp 285–293
9. Brown WJ, Goodhouse J, Farquhar MG: Mannose-6-phosphate receptors for lysosomal enzymes cycle between the Golgi complex and endosomes. J Cell Biol 103:1235–1247, 1986
10. Brumbaugh JA: The ultrastructural effects of I and S loci upon black–red melanin differentiation in the fowl. Devel Biol 24:392–412, 1971
11. Burchill SA, Thody AJ, Ito S: Melanocyte-stimulating hormone, tyrosinase activity and the regulation of eumelanogenesis and phaeomelanogenesis in the hair follicular melanocyte of the mouse. J Endocrinol 109:15–21, 1986
12. Endo H, Hu F: Pigment cell development in rhesus monkey eyes: an electron microscopic and histochemical study. Devel Biol 32:69–81, 1973
13. Eppig Jr JJ, Dumont JN: Cytochemical localization of tyrosinase activity in pigmented epithelial cells of *Rana pipiens* and *Xenopus* larvae. J Ultrastruct Res 39:397–410, 1972

14. Ferrini U, Mileo AM, Hearing VJ: Microheterogenicity of melanosome-bound tyrosinase from the Harding-Passey murine melanoma. Int J Biochem 19:227–234, 1987
15. Foulks JG: An analysis of the source of melanophores in regenerating feathers. Physiol Zool 16:351–380, 1943
16. Frenk E, Schellhorn JP: Zur Morphologie der epidermalen Melanineinheit. Dermatologica 139:271–277, 1969
17. Gilchrest BA, Blog FB, Szabo G: Effects of aging and chronic sun exposure on melanocytes in human skin. J Invest Dermatol 73:141–143, 1979
18. Glimcher ME, Kostick RM, Szabo G: The epidermal melanocyte system in newborn human skin: a quantitative histologic study. J Invest Dermatol 61:344–347, 1973
19. Goldgeier MH, Klein LE, Klein-Angerer S, et al: The distribution of melanocytes in the leptomeninges of the human brain. J Invest Dermatol 82:235–238, 1984
20. Halaban R, Pomerantz SH, Marshall S, et al: Regulation of tyrosinase in human melanocytes grown in culture. J Cell Biol 97:480–488, 1983
21. Hasilik A, Neufeld EF: Biosynthesis of lysosomal enzymes in fibroblasts: phosphorylation of mannose residues. J Biol Chem 255:4946–4950, 1980
22. Hearing VJ, Ekel TM, Montague PM: Mammalian tyrosinase: isozymic forms of the enzyme. Int J Biochem 13:99–103, 1981
23. Hearing VJ Jr, Ekel TM, Montague PM, et al: Mammalian tyrosinase: isolation by a simple new procedure and characterization of its steric requirements for cofactor activity. Arch Biochem Biophys 185:407–418, 1978
24. Honigsmann H, Schuler G, Aberer W, et al: Immediate pigment darkening phenomenon: a reevaluation of its mechanisms. J Invest Dermatol 87:648–652, 1986
25. Hu F, Mah K: Choroidal melanocytes: a model for studying the aging process in nonreplicative differentiated cells. Mech Aging Dev 11:227–235, 1979
26. Hu F, Mah K: Changes in melanosomes with age in iridial stroma melanocytes of rhesus macaques. Mech Aging Dev 23:95–102, 1983
27. Hu F, Montagna W: The development of pigment cells in the eyes of rhesus monkeys. Am J Anat 132:119–132, 1971
28. Ideda S, Makita A, Tajima M, et al: Clinical and histologic study of blue nevus. In Fitzpatrick TB, et al (eds): Biology and Diseases of Dermal Pigmentation. Tokyo, University of Tokyo Press, 1981, pp 95–106
29. Imokawa G, Mishima Y: Functional analysis of tyrosinase isozymes of cultured malignant melanoma cells during the recovery period following interrupted melanogenesis induced by glycosylation inhibitors. J Invest Dermatol 83:196–201, 1984
30. Imokawa G, Mishima Y: Importance of glycoproteins in the initiation of melanogenesis: an electronmicroscopic study of B-16 melanoma cells after release from inhibition of glycosylation. J Invest Dermatol 87:319–325, 1986
31. Jimbow K, Fitzpatrick TB: Changes in distribution pattern of cytoplasmic filaments in human melanocytes during ultraviolet-mediated melanin pigmentation: the role of 100 Å filaments in the elongation of melanocytic dendrites and in the movement and transfer of melanosomes. J Cell Biol 65:481–488, 1975
32. Jimbow K, Fitzpatrick TB: Characterization of a new melanosomal structure component—the vesiculo-globular body—by conventional transmission, high-voltage and scanning electric microscope. J Ultrastruct Res 48:269–283, 1974
33. Jimbow K, Oikawa O, Segiyama S, et al: Comparison of eumelanogenesis and pheomelanogenesis in retinal and follicular melanocytes: role of vesiculo-globular bodies in melanosome differentiation. J Invest Dermatol 73:278–384, 1979
34. Jimbow, K, Pathak MA, Szabo G, et al: Ultrastructural changes in human melanocytes after ultraviolet radiation. In Fitzpatrick TB (ed): Sunlight and Man. Tokyo, University of Tokyo Press, 1974, pp 195–216
35. Jimbow K, Roth SI, Fitzpatrick TB, et al: Mitotic activity in non-neoplastic melanocytes in vivo as determined by histochemical, autoradiographic and electron microscope studies. J Cell Biol 66:663–670, 1975
36. Jimbow K, Takahashi M, Sato S, et al: Ultrastructural and cytochemical studies of melanogenesis in melanocytes of normal human hair matrix. J Electron Microsc 20:87–92, 1971
37. Jimbow M, Kanoh H, Jimbow K: Characterization of biochemical properties of melanosomes for structural and functional differentiation: analysis of the compositions of lipids and proteins in melanosomes and their subfractions. J Invest Dermatol 79:97–102, 1982
38. Kikuchi I, Inoue S: Circumscribed dermal melanocytosis (Mongolian spot). In Fitzpatrick TB, et al (eds): Biology and Diseases of Dermal Pigmentation. Tokyo, University of Tokyo Press, 1981, pp 83–94
39. Konrad K, Woiff K: Hyperpigmentation, melanosome-size and distribution patterns of melanosomes. Arch Dermatol 107:853–860, 1973
40. Korner A, Pawelek J: Mammalian tyrosinase catalyzes three reactions in the biosynthesis of melanin. Science 217:1163–1165, 1982
41. Kukita A, Hori Y, Ohhara K, et al: Nevus of Ota. In Fitzpatrick JB, et al (eds): Biology and Diseases of Dermal Pigmentation. Tokyo, University of Tokyo Press, 1981, pp 67–76
42. LeDourain NM, Teillet MA: Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neuroectodermal mesenchymal derivatives using a biological cell marking technique. Dev Biol 41:162–184, 1974
43. Lerner AB, Fitzpatrick TB: Biochemistry of melanin formation. Physiol Rev 30:91–126, 1950
44. Loring J, Climeluis B, Erickson C, et al: Analysis of developmentally homogenous neural crest cell population *in vitro* 1: formation, morphology and differentiative behavior. Dev Biol 82:86–94, 1981
45. Masson P: Pigment cells in man. Special Publ NY Acad Sci 4:15–51, 1948
46. Maul GG: Golgi–melanosome relationship in human melanoma *in vitro*. J Ultrastruct Res 26:163–176, 1969
47. McGuire J, Muellmann G: Cytochalasin B: effects on microfilaments and movement of melanin granules within melanocytes. Science 175:642–644, 1972
48. NcNiven MA, Porter KR: Microtubule polarity confers direction to pigment transport in chromatophores. J Cell Biol 103:1547–1555, 1986
49. Mellman I, Fuchs R, Helenius A: Acidification of the endocytic and exocytic pathways. Annu Rev Biochem 55:663–700, 1986
50. Mishima Y, Imokawa G: Selective aberration and pigment loss in melanosomes of malignant melanoma cells in vitro by glycosylation inhibitors: premelano-

somes as glycoprotein. J Invest Dermatol 81:106–114, 1983
51. Mishima Y, Imokawa G: Role of glycosylation in initial melanogenesis: post-inhibition dynamics. In Bagnara J, Klaus SN, Paul E, et al (eds): Pigment Cell, 1985: Proceedings of the XIIth International Pigment Cell Conference, Giessen, Germany, 1983. Tokyo, University of Tokyo Press, 1985, pp 17–30
52. Mishima Y, Widlan S: Embryonic development of melanocytes in human hair and epidermis: their cellular differentiation and melanogenic activity. J Invest Dermatol 46:263–277, 1966
53. Moellmann G, McGuire J, Lerner AB: Ultrastructure and cell biology of pigment cells: intracellular dynamics and the fine structure of melanocytes with special reference to the effects of MSH and cyclic AMP on microtubules and 10 nm filaments. Yale J Biol Med 46:337–360, 1973
54. Mottaz JH, Zelickson AS: Ultrastructure of hair pigment. In Montagna W, Dobson RL (eds): Advances in Biology of Skin. London, Pergamon Press, 1969, pp 471–489
55. Nakagawa H, Rhodes AR, Montaz TK, et al: Morphologic alterations of epidermal melanocytes and melanosomes in PUVA lentigines: a comparative ultrastructural investigation of lentigines induced by PUVA and sunlight. J Invest Dermatol 82:101–107, 1984
56. Nordlund JJ: The lives of pigment cells. Dermatol Clin 4:407–418, 1986
57. Nordlund JJ: Inflammation and melanocytes: possible mechanisms of post inflammatory hypo- and hypermelanosis. In Bagnara JT (ed): Pigment Cell 1986: Proceedings of the XIIIth International Pigment Cell Conference, Tucson, Arizona. New York, Alan R Liss (in press)
58. Novikoff AB, Albala A, Biempica L: Ultrastructural and cytochemical observations on B-16 and Harding-Passey mouse melanomas: the origin of premelanosomes and compound melanosomes. J Histochem Cytochem 16:299–319, 1968
59. Ohkura T, Yamashita K, Mishima Y, et al: Purification of hamster melanoma tyrosinases and structural studies of their asparagine-linked sugar chains. Arch Biochem Biophys 235:63–77, 1984
60. Orentreich N: Hair problems. J Am Med Womens Assoc 21:481–486, 1966
61. Pawelek J, Körner A, Bergstrom A, et al: New regulators of melanin biosynthesis and the autodestruction of melanoma cells. Nature 286:617–619, 1980
62. Pomerantz SH: L-tyrosine-3,5-^{3}H assay for tyrosinase development in skin of newborn hamsters. Science 164:838–839, 1969
63. Prota G: Recent advances in the chemistry of melanogenesis in mammals. J Invest Dermatol 75:122–127, 1980
64. Quevedo WC Jr: Comparative biology of dermal pigmentation in mammals. In Fitzgerald TB, et al (eds): Biology and Diseases of Dermal Pigmentation. Tokyo, University of Tokyo Press, 1981, pp 67–76
65. Rawles ME: The migration of melanoblasts after hatching into pigment-free skin grafts of the common fowl. Physiol Zool 17:167–183, 1944
66. Rawles M: Origin of pigment cell from the neural crest in the mouse embryo. Physiol Zool 20:248–266, 1947
67. Reams WM Jr: Morphogenesis of pigment cells in the connective tissue of the PET mouse. Ann NY Acad Sci 100:486–495, 1963
68. Reams WM Jr, Shervette RE, Dorman WH: Refractoriness of mouse dermal melanocytes to hormones. J Invest Dermatol 50:338–339, 1968
69. Rhodes AR, Harrist TJ, Montaz TK: The PUVA-induced pigmented macule: a lentiginous proliferation of large, sometimes cytologically atypical, melanocytes. Am Acad Dermatol 9:47–58, 1983
70. Saeki H, Oikawa A: Stimulation by ionophores of tyrosinase activity of mouse melanoma cells in culture. J Invest Dermatol 85:423–425, 1985
71. Sakurai T, Ochiai H, Takeuchi T: Ultrastructural change of melanosomes associated with agouti pattern formation in mouse hair. Dev Biol 47:466–471, 1975
72. Seiji M, Toda K, Okazaki K, et al: Melanocyte–keratinocyte interaction in pigment transfer. In Riley V (ed): Pigment Cell, vol 3. Basel, Karger, 1976, pp 393–405
73. Sugiyama S: Mode of redifferentiation and melanogenesis of melanocytes in mouse hair follicles: an ultrastructural and cytochemical study. J Ultrastruct Res 67:40–54, 1979
74. Sugiyama S, Kukita A: Melanocyte reservoir in the hair follicles during the hair growth cycle: an electron microscopic study. In Kobori T, Montagna W (eds): Biology and Diseases of the Hair. Tokyo, University of Tokyo Press, 1976, pp 181–200
75. Szabo G: Photobiology of melanogenesis: cytological aspects with special reference to differences in racial coloration. In Advances in Biology of Skin, volume 8: The Pigmentary System. New York, Pergamon Press, 1967, pp 379–396
76. Szabo G, Gerald AB, Pathak MA, et al: Racial differences in the fate of melanosomes in human epidermis. Nature 222:1081–1082, 1969
77. Toda K, Matsumoto J, Fitzpatrick TB: Structure of melanosomes in human red hair bulbs. In Kobori T, Montagna W (eds): Biology and Diseases of the Hair. Tokyo, University of Tokyo Press, 1976, pp 217–228
78. Toda K, Pathak MA, Parrish JA, et al: Alteration of racial differences in melanosome distribution in human epidermis after exposure to ultraviolet light. Nature New Biol 236:143–145, 1972
79. Toda K, Pathak MA, Fitzpatrick TB, et al: Skin color: its ultrastructure and its determining mechanism. In Riley V (ed): Pigment Cell: Proceedings of the 8th International Pigment Cell Conference, Sydney. Basal, S Karger, 1973, pp 66–81
80. Topol BM, Haimes HB, Dubertret L, et al: Transfer of melanosomes in a skin equivalent model in vitro. J Invest Dermatol 87:642–647, 1986
81. Turner WA Jr, Taylor JD, Chen TT: The role of multivesicular bodies in melanosome formation. In Riley V: Pigment Cell, vol 13: Proceedings of the 9th International Pigment Cell Conference, Houston, Texas, 1975. Basal, S Karger, 1976, pp 33–45
82. Weston JA: The migration and differentiation of neural crest cells. Adv Morphol 8:41–114, 1970
83. Wolff K, Kimbow K, Fitzpatrick TB: Experimental pigment donation *in vivo*. J Ultrastruct Res 47:400–419, 1974
84. Wolff K, Konrad K: Melanin pigmentation: an in vivo model for studies of melanosome kinetics within keratinocytes. Science 174:1034–1035, 1971

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THE

PIGMENTARY SYSTEM

Physiology and Pathophysiology

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New York Oxford
OXFORD UNIVERSITY PRESS
1998

the melanocytes in the leptomeninges differs from the dark pigment found within neurons throughout the brain. The latter is classified as neuromelanin and is thought to be an auto-oxidative product(s) of dopamine generated by the neuronal synthesized tyrosine hydroxylase molecule (see Breathnach, 1988; Enochs *et al.*, 1993, 1994; Odh *et al.*, 1994; Zecca *et al.*, 1992; see also Chapter 25). The function of the melanocytes confined to the leptomeninges is not clear. It has been speculated that these melanocytes may be affected in patients with severe vitiligo (i.e., the Vogt-Koyanagi-Harada syndrome) and that their loss may correlate with the aseptic meningitis component of this syndrome (Goldgeier *et al.*, 1984; Nordlund *et al.*, 1980).

Melanocytes in the harderian gland

The harderian gland is a secretory structure located at the medial back of the optic orbit with a duct that connects it to the nictitating membrane of the eyelid (Payne, 1994). It exists in all groups of terrestrial vertebrates but is absent in some types of mammals (i.e., bats, cows and horses), terrestrial carnivores, and higher primates including humans. Structurally, the gland is composed of tubules of simple, columnar epithelial cells surrounded by myoepithelial cells and it primarily synthesizes lipids, porphyrins, and indoles. Many putative and diverse functions have been hypothesized for this secretory gland (Payne, 1994). Melanocytes have been identified in the harderian glands of mice (Shirama *et al.*, 1988), gerbils (Johnston *et al.*, 1983), and frogs (Di Matteo *et al.*, 1989). These melanocytes are primarily localized throughout the connective tissue capsule surrounding the harderian gland and intermittently among the connective tissue septa invaginations into the gland that separate the stroma into lobules (Fig. 4–6). Like the melanocytes of the leptomeninges, the melanocytes in the harderian gland of an adult animal are multidendritic, melanogenically silent, and congested with fully pigmented melanosomes (Fig. 4–6). Harderian glands ultrastructurally observed from the murine hypopigmented mutants ruby eye (*Ru/Ru*) and dilute (*di/di*) exhibited relatively fewer melanocytes with a dramatic reduction in melanosomes distributed throughout the cytoplasm (R.E. Boissy & K. Moore, unpublished observations). The function of melanocytes in the harderian gland is unknown and no dysfunction in any of the putative roles of the gland has been reported in association with the various murine hypopigmentary mutants.

Melanocytes in mesenchymal tissues

There have been a few reports of melanocytes distributed throughout internal body sites, most notably related to the PET mouse (Reams, 1963) and the Silkie chicken (Stolle, 1968).

In the PET mouse, melanocytes appearing multidendritic, heavily pigmented, and inactive are distributed throughout the connective tissues of multiple sites in the body cavity including cartilage, bone, serosae, lungs, heart, gonads, etc. (Nichols & Reams, 1960; Rovee & Reams, 1964). The specific genetics of this undefined strain of mouse has not been clearly described, however, the inheritance of the mesenchymal pigmentation in the PET mouse appears to have been autosomal recessive in nature (Mayer & Reams, 1962; Reams, 1967).

In the Silkie chicken, melanocytes have been identified in the loose connective tissue throughout the body and associated with the connective tissue coverings of cartilage (perichondrium) and bone (periostium). In addition, melanocytes are prevalent in the gonads, lungs, thymus gland, thyroid gland, spinal ganglia, and gray matter of the spinal cord but absent from the liver, pancreas, and spleen (Hallet & Ferrand, 1984; Makita & Mochizuki, 1984; Makita & Tsuzuki, 1986; Stolle, 1968). This mesenchymal distribution of melanocytes is primarily the result of an autosomal dominant gene (*P*) which can be intensified by a sex-linked recessive gene (*d*) (Stolle, 1968). Current analysis of the migration pathway of neural crest–derived melanoblasts in Silkie embryos has demonstrated that these precursors are not restricted to the dorsolateral pathway as in normal development but also aberrantly migrate through the ventral pathway (Reedy & Erickson, 1995). This genetically controlled diversion of melanoblast migration appears to be under cues extrinsic to this neural crest derivative (Hallet & Ferrand, 1984) (see Chapter 5).

PERSPECTIVES

Extracutaneous melanocytes found in the eye, ear, and various internal body structures are distinct as a whole from epidermal melanocytes in that (*1*) they synthesize melanosomes and melanin only during a short interval after arriving in their target tissue, and (2) they retain their melanosomes throughout the life of the organism instead of transferring them to neighboring cells. The general morphology and ultrastructure of these extracutaneous melanocytes and the specific variations that exist among them have been well documented in the literature. However, the role of these morphologically dormant melanocytes in their respective tissues has primarily been ascertained anecdotally to date. Fortunately, experimental analysis investigating the function of extracutaneous melanocytes is beginning to make a reappearance in the scientific literature. The advent of molecular genetic technology and its combination with cellular and physiological approaches will help resolve the elusive function of these relatively ignored populations of melanocytes which have been retained throughout evolution.

Summary

1. Melanocytes that occur in ocular tissue are subdivided into two categories, uveal melanocytes (found in the stroma of the choroid, ciliary bodies, and iris) and retinal pigment epithelium (RPE) melanocytes. During embryogenesis, uveal melanocytes are derived from neural crest cells that emigrate from the neural tube and migrate to the developing eye tissue, whereas RPE melanocytes differentiate *de novo* from a cephalic portion of the neural tube that forms the neural retina.

2. Uveal and RPE melanocytes differ structurally from each other. Uveal melanocytes are multidendritic and synthesize melanosomes relatively similar in shape. RPE melanocytes are highly polarized in structure and synthesize melanosomes with variable shape. Both categories of melanocytes complete the process of melanization shortly after birth and are melanogenically silent throughout the remaining life of the organism.

3. Ocular melanocytes appear essential for the complete development of both the neural retina and the neuronal network to the visual center of the brain as exemplified in piebaldism/microphthalmia and albinism, respectively. Ocular melanocytes and the melanin they contain function to absorb incident light that passes through the photoreceptor cells, transfer nutrients and wastes between the retina and the vascular system of the eye, and phagocytize the outer segments of the photoreceptor cells as they are shed.

SURVEY OF OPHTHALMOLOGY VOLUME 49 • NUMBER 4 • JULY–AUGUST 2004

ELSEVIER

PUBLIC HEALTH AND THE EYE

JOHANNA SEDDON AND DONALD FONG, EDITORS

Sunlight Exposure and Pathogenesis of Uveal Melanoma

Arun D. Singh, MD, FRCS, FRCOphth,[1,2] Ian G. Rennie, FRCS, FRCOphth,[2] Stefan Seregard, MD,[3] Michael Giblin, MD,[4] and John McKenzie, MD[5]

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Abstract. Uveal melanoma is the most frequent primary malignant intraocular tumor of adults. Among various non-modifiable risk factors, Caucasian race seems to be the most significant with light skin color, blond hair, and blue eyes being specific risk factors. The racial predisposition to uveal melanoma have been explained on the basis of susceptibility of Caucasian race to oncogenic effects of sunlight. Although there is ample evidence in support of this hypothesis in regard to skin melanoma, the evidence in regard to uveal melanoma is insufficient and contradictory. In the following review, we examine physiologic, epidemiological, and genetic data in order to determine the role of sunlight exposure in the pathogenesis of uveal melanoma. (**Surv Ophthalmol** 49:419–428, 2004. © 2004 Elsevier Inc. All rights reserved.)

Key words. melanoma • sunlight • ultraviolet light • uvea • uveal melanoma

Uveal melanoma is the most frequent primary malignant intraocular tumor of adults but represents only 3% of all melanoma.[13,77] Despite numerous epidemiological studies the etiology of uveal melanoma remains obscure.[13] Among various host factors that have been identified to render predisposition, race seems to be the most significant, as uveal melanoma is about 150 times more common in white than in black individuals.[13,23,77] In addition, uveal melanoma is less common in Asians.[6,36,42] Among the Caucasian race, light skin color,[22,57] blond hair,[17] and blue eyes,[17,22,32,38,57,65,85] are specific risk factors. The racial predisposition to uveal melanoma has been explained on the basis of susceptibility of Caucasian race to oncogenic effects of sunlight.

There is strong epidemiologic evidence linking increased exposure to sunlight with increasing incidence of skin melanoma.[20] The incidence of skin melanoma is much higher in white individuals than in those with darker skin color. In the United States, the incidence is 10 times greater in Caucasians than in black individuals.[59] The epidemiologic evidence is further corroborated by the biologic evidence of ultraviolet light induced DNA damage in the pathogenesis of skin melanoma.[15,16]

Altough there is ample evidence in support of sunlight exposure in regard to pathogenesis of skin

0039-6257/04/$–see front matter
doi:10.1016/j.survophthal.2004.04.009

exerts a photo-protective role by absorbing UV light and reactive oxygen species generated by the interaction of UV light and membrane lipids.[64] Within cells, melanin is distributed in supranuclear caps that protects the underlying nucleus from UV light injury.[39]

Cornea

The cornea effectively filters UV light below 295 nm and transmits most UV-A and UV-B light. With increasing age there is reduction in percent transmission of light through cornea across the visible and UV light spectrum, but the decline in transmission of UV-A light and UV-B light is most pronounced (Fig. 2).[7,44]

Lens

The lens also acts as a filter of UV light for wavelengths between 300 nm and 400 nm. However, there is a small transmission window around 320 nm that closes around 10 years of age due to progressive accumulation of lenticular chromatophores.[63,95,96] In early childhood the lens transmits about 75% of UV light, which declines to less than 20% by 80 years of age (Fig. 3).[45]

Retinal Pigment Epithelium

About 30% of remaining UV light is absorbed by the retinal pigment epithelium before impinging upon the choroidal melanocytes (Fig. 4).[19,60] The posterior pole of the eye, which is exposed maximally to the transmitted light, has the highest density of the retinal pigmented epithelial cells and thus provides the greatest amount of absorption.[56]

SITES OF UVEAL MELANOMA ORIGIN

In an attempt to analyze patterns of tumor initiation in relation to the intraocular light distribution,

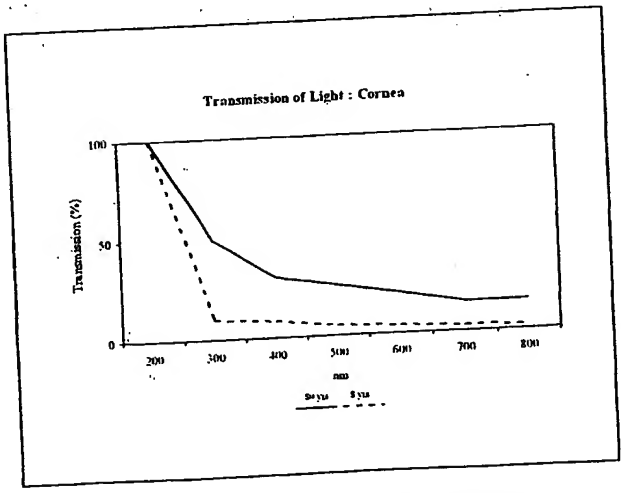

Fig. 2. Transmission of light by cornea. (Modified from Lerman.[43–45])

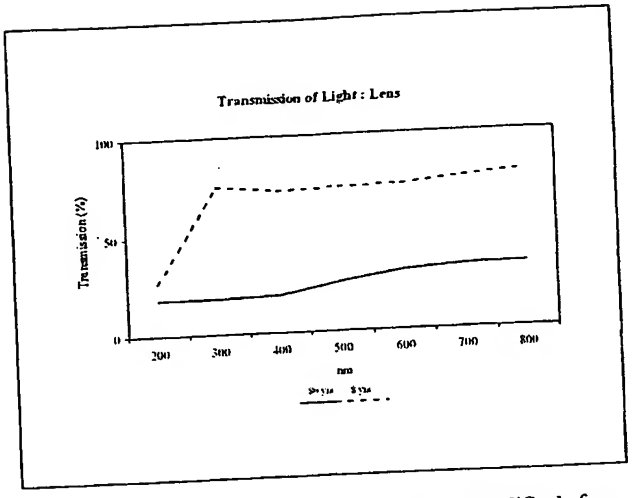

Fig. 3. Transmission of light by lens. (Modified from Lerman.[43–45])

a study was conducted where the tumor initiation site was defined as the intersection of the largest tumor diameter and the largest perpendicular diameter of the tumor. The "initiation sites" were recorded using spherical coordinates.[46] Findings suggested that the site of tumor origin was not uniformly distributed in the choroid with predilection for macular region. Moreover, the density of occurrence decreased progressively with increasing distance from the macula. The distribution of tumor origin correlated with the dose distribution of solar irradiation on the retinal sphere.[46] These findings contradict a previous report in which the distribution of tumors in the choroid was randomly distributed.[72]

Even if the site of tumor origin in the choroid correlates with the distribution of solar light on the retinal sphere, this model fails to explain the occurrence of melanoma in the ciliary body as only negligible amount of the UV light reaches this part of the uvea.

Epidemiological Studies

The relationship of sunlight and UV light exposure and occurrence of uveal melanoma has been evaluated in a few case-control studies (Tables 1 and 2). The case-control studies have inherent methodological limitations as such studies rely on questionnaires about lifestyle and exposures that occurred many years before the onset of disease (in this case uveal melanoma) and are subject to recall bias.[71] Recall bias probably occurs because persons with a given disease are prone to think about and reflect on the cause of their condition much more than persons unaffected by the disease. Patients may also be more familiar with unproven hypotheses (such as UV light and causation of uveal melanoma) than individuals in the general population who do not have the disease.

TABLE 1

Summary of Results from Case Control Studies Evaluating Risk Factors in Uveal Melanoma

First Author	Year	Region	Number of Cases	Risk Factors: Significant — Positive Correlation	Risk Factors: Significant — Negative Correlation	Risk Factors: Insignificant
Gallagher[17]	1985	Western Provinces (Canada)	90	Red/Blonde Hair Color (OR = 7.7, p = 0.03) Indoor Workers (OR = 3.5, p = 0.006) Blue Eyes (OR = 3.0, p = 0.04)		Sunlight Exposure Tanning Ability
Tucker[85]	1985	United States	444	Born in South (OR = 2.7 [1.3–5.9]) Sunbathing (OR = 1.5 [0.9–2.3]) Use of Sunlamps (OR = 2.1 [0.3–17.9])	Brown Eyes	Complexion Hair Color
Seddon[75]	1990	New England (United States)	197	Northern European Ancestry (OR = 6.5 [1.9–22.4]) Light Skin Color (OR = 3.8 [1.1–12.6]) Use of Sunlamps (OR = 3.4 [1.1–10]) Sun Exposure (OR = 1.7 [0.9–3.0])	Outdoor Activity Born in South	
Holly[26]	1990	Western (United States)	407	Welding Burn/Snow Blindness (OR = 7.2, p < 0.001) Light Colored Eyes (OR = 2.5, p < 0.001) Exposure to UV light (OR = 3.7, p = 0.003) Tendency to Sunburn (OR = 1.8, p < 0.001)		
Pane[57]	2000	Queensland (Australia)	125	History of Skin Melanoma (OR = 2.42 [0.88–6.62]) Family History of Ocular Melanoma (OR = 6.89 [0.70–67.38])	Dark Skin Color Brown Eye Resistance to Sunburn Wearing Prescription Glasses	Wearing Sunglasses Lifetime Ocular UV-B Exposure
Vadjic[89]	2002	Australia	290	Outdoors Activity (OR = 1.8 [1.1–2.8])[a]		

OR = Odds ratio [95% Confidence Interval].

[a]Sun exposure was an independent risk factor for choroid and ciliary body melanoma but not iris or conjunctival melanomas.

the group using electric arc welding.[22] A larger study from France, which analyzed 412 patients with uveal melanoma, found no statistically significant association with occupation.[90] In a detailed case-control study of 197 uveal melanoma patients, in which subjects and controls were interviewed regarding occupational history and ultraviolet light exposure, no positive associations were reported.[2] At present, there is no consistent evidence indicating occupational UV light exposure as a risk factor for uveal melanoma.

INCIDENCE OF UVEAL MELANOMA

Temporal Stability

The mean age-adjusted incidence of uveal melanoma of 4.3 per million/year was reported based on a systematic review of the Surveillance, Epidemiology, and End Result (SEER) program database of the United States.[77] The age-adjusted annual incidence rate of uveal melanoma in the United States has remained stable from 1973 to 1997. In Sweden, the

TABLE 3

Summary of Published Reports on Incidence of Uveal Melanoma

First Author	Period	Region	Definition	Incidence Rate/Per Million
Mork[54]	1953–1960	Norway	Ocular melanoma	9.0
Jensen[32]	1943–1952	Denmark	Uveal melanoma	7.4
Ganley[18]	1956–1965	Maryland (US)	Choroidal melanoma	6.6
Scotto[74]	1969–1971	United States	Eye melanoma	5.6
Raivio[62]	1953–1973	Finland	Cbd+ choroid	5.3
Shammas[76]	1969–1971	Iowa (US)	Cbd+ choroid	4.9 (White)
Davidorf[10]	1967–1977	Ohio (US)	Choroidal melanoma	10.9 (White adults)
Wilkes[94]	1935–1974	Rochester (US)	Uveal melanoma	7.0
Birdsell[5]	1967–1977	Alberta (Canada)	Eye melanoma	6.0
Strickland[80]	1950–1974	Connecticut (US)	Eye melanoma	9.0 (Male), 8.0 (Female)
Kaneko[36]	1977–1979	Japan	Uveal melanoma	0.3
Abrahamsson[1]	1956–1975	Halland (Sweden)	Cbd+ choroid	7.2
Swerdlow[82]	1952–1978	Oxford (UK)	Ocular melanoma	4.8 (Male), 3.9 (Female)
Swerdlow[81]	1962–1977	England (UK)	Ocular melanoma	7.2 (Male), 5.7 (Female)
Gislason[21]	1955–1979	Iceland	Cbd+ choroid	7.0 (Male), 5.0 (Female)
Lommatzsch[47]	1961–1980	East Germany	Eye melanoma	10.0
Teikari[83]	1973–1980	Finland	Cbd+ choroid	7.6
Egan*	1984	New England (US)	Uveal melanoma	6.9
Iscovich[31]	1961–1989	Israel	Cbd +Choroid	5.7 (Jews)
Vidal[90]	1992	France	Uveal melanoma	7.0
Margo[48]	1981–1983	Florida(US)	Uveal melanoma	5.6
Bergman[4]	1960–1989	Sweden	Uveal melanoma	9.4 (Male), 8.8 (Female)
Singh[77]	1973–1997	United States	Uveal melanoma	4.9 (Male), 3.7 (Female)
Kricker[88]	1996–1998	Australia	Choroidal melanoma	11.0 (Male), 7.8 (Female)

Uveal melanoma = Iris, ciliary body (Cbd), and choroidal melanoma; Eye melanoma = Uveal and conjunctival melanoma; Ocular melanoma = Uveal, conjunctival, and eyelid melanoma.

*(Source Egan K, Seddon JM, Grogoudas PS, et al: Uveal melanoma in New England: profile of cases diagnosed in 1984 [abstract]. Invest Ophthalmol Vis Sci 28:144,1987)

Biological Variants

XERODERMA PIGMENTOSUM

Xeroderma pigmentosum is an autosomal recessive, precancerous disorder due to defective nucleotide excision repair mechanism. The nucleotide excision repair mechanism specifically protects against mutations caused by environmental carcinogens such as UV light. Xeroderma pigmentosum serves as a model for ultraviolet light–induced carcinogenesis.[16]

In a review of published descriptions of 830 patients with xeroderma pigmentosum,[41] 45% of the patients had basal cell carcinoma or squamous cell carcinoma of the skin and 5% of the patients had cutaneous melanoma.[40,41] The ocular neoplasms in xeroderma pigmentosum are of surface squamous cell type rather than uveal melanoma.[3,35,41] If sunlight had a major role in pathogenesis of uveal melanoma or if xeroderma pigmentosum was a genetic predisposing factor,[78] more cases of uveal melanoma in xeroderma pigmentosum would be expected.

ALBINISM

Patients with albinism are extremely sensitive to ultraviolet light due to lack of protective melanin. Such patients are susceptible to frequent occurrences of squamous cell carcinomas and basal cell carcinoma.[27] At least two cases of uveal melanoma in albinism have been reported. One occurred in the nonpigmented choroid of a tyrosinase-positive oculocutaneous albino,[9] and the other in an African-American albino.[37] Given the frequency of Albinism and the paucity of case reports with uveal melanoma, UV light would appear to have limited role in the pathogenesis of uveal melanoma.

Oncogenicity of Ultraviolet Light

The incidence of cutaneous melanoma has increased about 15 times over the last 60 years.[58] There is strong epidemiological evidence that increase in the incidence of cutaneous melanoma is related to excessive intermittent exposure to the sunlight. A systematic review of published case-control studies revealed a significant positive association (OR = 1.71) for intermittent sun exposure but a weaker association for total sun exposure (OR = 1.18).[14] Exposure to high levels of sunlight in childhood may be more carcinogenic than sun exposure in adulthood.[92] Primary carcinogenic effect of sunlight is attributed to UV-B light (280–320 nm)

30. Iscovich J, Abdulrazik M, Pe'Er J: Posterior uveal malignant melanoma: temporal stability and ethnic variation in rates in Israel. Anticancer Res 21:1449–54, 2001
31. Iscovich J, Ackerman C, Andreev H, et al: An epidemiological study of posterior uveal melanoma in Israel, 1961–1989. Int J Cancer 61:291–5, 1995
32. Jensen O: Malignant melanomas of the uvea in Denmark 1943–1952. Acta Ophthalmol 75:17–78, 1963
33. Jensen OA: Malignant melanoma of the iris. A 25-year analysis of Danish cases. Eur J Ophthalmol 3:181–8, 1993
34. Jiveskog S, Ragnarsson-Olding B, Platz A, Ringborg U: N-ras mutations are common in melanomas from sun-exposed skin of humans but rare in mucosal membranes or unexposed skin. J Invest Dermatol 111:757–61, 1998
35. Johnson MW, Skuta GL, Kincaid MC, et al: Malignant melanoma of the iris in xeroderma pigmentosum. Arch Ophthalmol 107:402–7, 1989
36. Kaneko A: [Malignant ophthalmic tumors]. Nippon Rinsho 51(Suppl):1013–20, 1993
37. Kheterpal S, Shields JA, Shields CL, et al: Choroidal melanoma in an African-American albino. Am J Ophthalmol 122:901–3, 1996
38. Kliman GH, Augsburger JJ, Shields JA: Association between iris color and iris melanocytic lesions. Am J Ophthalmol 100:547–8, 1985
39. Kobayashi N, Nakagawa A, Muramatsu T, et al: Supranuclear melanin caps reduce ultraviolet induced DNA photoproducts in human epidermis. J Invest Dermatol 110:806–10, 1998
40. Kocabalkan O, Ozgür F, Erk Y, et al: Malignant melanoma in xeroderma pigmentosum patients: report of five cases. Eur J Surg Oncol 23:43–7, 1997
41. Kraemer KH, Lee MM, Scotto J: Xeroderma pigmentosum. Cutaneous, ocular, and neurologic abnormalities in 830 published cases. Arch Dermatol 123:241–50, 1987
42. Kuo PK, Puliafito CA, Wang KM, et al: Uveal melanoma in China. Int Ophthalmol Clin 22:57–71, 1982
43. Lerman S: Radiant energy and the eye. New York, Macmillan Publishing Co., Inc., 1980, pp 2–3
44. Lerman S: Radiant energy and the eye. New York, Macmillan Publishing Co., Inc., 1980, pp 58
45. Lerman S: Radiant energy and the eye. New York, Macmillan Publishing Co., Inc., 1980, pp 88
46. Li W, Judge H, Gragoudas ES, et al: Patterns of tumor initiation in choroidal melanoma. Cancer Res 60:3757–60, 2000
47. Lommatzsch PK, Staneczek W, Bernt H: [Epidemiologic study of new cases of intraocular tumors in East Germany 1961–1980]. Klin Monatsbl Augenheilkd 187:487–92, 1985
48. Margo CE, Mulla Z, Billiris K: Incidence of surgically treated uveal melanoma by race and ethnicity. Ophthalmology 105: 1087–90, 1998
49. McKenzie R, Connor B, Bodeker G: Increased summertime UV radiation in New Zealand in response to ozone loss. Science 285:1709–11, 1999
50. Menon IA, Wakeham DC, Persad SD, et al: Quantitative determination of the melanin contents in ocular tissues from human blue and brown eyes. J Ocul Pharmacol 8:35–42, 1992
51. Michalova K, Clemett R, Dempster A, et al: Iris melanomas: are they more frequent in New Zealand? Br J Ophthalmol 85:4–5, 2001
52. Mitchell DL, Jen J, Cleaver JE: Relative induction of cyclobutane dimers and cytosine photohydrates in DNA irradiated in vitro and in vivo with ultraviolet-C and ultraviolet-B light. Photochem Photobiol 54:741–6, 1991
53. Mooy CM, Van der Helm MJ, Van der Kwast TH, et al: No N-ras mutations in human uveal melanoma: the role of ultraviolet light revisited. Br J Cancer 64:411–3, 1991
54. Mork T: Malignant melanoma of the eye in Norway: incidence, treatment and prognosis. Acta Ophthalmol 39:824–31, 1961
55. Ortonne JP: Photobiology and genetics of malignant melanoma. Br J Dermatol 61(Suppl):11–6, 2002
56. Panda-Jonas S, Jonas JB, Jakobczyk-Zmija M: Retinal pigment epithelial cell count, distribution, and correlations in normal human eyes. Am J Ophthalmol 121:181–9, 1996
57. Pane AR, Hirst LW: Ultraviolet light exposure as a risk factor for ocular melanoma in Queensland, Australia. Ophthalmic Epidemiol 7:159–67, 2000
58. Parker SL, Tong T, Bolden S, Wingo PA: Cancer statistics, 1997. CA Cancer J Clin 47:5–27, 1997
59. Parkin DM, Muir CS, Whelan SL, et al (eds): Cancer incidence in five continents. Lyon, France, International Agency for Research on Cancer, 1992
60. Peyman GA, Raichand M, Zeimer RC: Ocular effects of various laser wavelengths. Surv Ophthalmol 28:391–404, 1984
61. Ragnarsson-Olding B, Johansson H, Rutqvist LE, Ringborg U: Malignant melanoma of the vulva and vagina. Trends in incidence, age distribution, and long-term survival among 245 consecutive cases in Sweden 1960–1984. Cancer 71:1893–7, 1993
62. Raivio I: Uveal melanoma in Finland. An epidemiological, clinical, histological and prognostic study. Acta Ophthalmol 133(Suppl):1–64, 1977
63. Remé C, Reinboth J, Clausen M, Hafezi F: Light damage revisited: converging evidence, diverging views? Graefes Arch Clin Exp Ophthalmol 234:2–11, 1996
64. Riley PA: Melanin. Int J Biochem Cell Biol 29:1235–9, 1997
65. Rootman J, Gallagher RP: Color as a risk factor in iris melanoma. Am J Ophthalmol 98:558–61, 1984
66. Rosenthal FS, Bakalian AE, Lou CQ, Taylor HR: The effect of sunglasses on ocular exposure to ultraviolet radiation. Am J Public Health 78:72–4, 1988
67. Rosenthal FS, Bakalian AE, Taylor HR: The effect of prescription eyewear on ocular exposure to ultraviolet radiation. Am J Public Health 76:1216–20, 1986
68. Rosenthal FS, Phoon C, Bakalian AE, Taylor HR: The ocular dose of ultraviolet radiation to outdoor workers. Invest Ophthalmol Vis Sci 29:649–56, 1988
69. Rosenthal FS, West SK, Munoz B, et al: Ocular and facial skin exposure to ultraviolet radiation in sunlight: a personal exposure model with application to a worker population. Health Phys 61:77–86, 1991
70. Rosner B: Fundamentals of biostatistics. Belmont, CA, Wadsworth Publishing Company, 1995, pp 367–70
71. Sackett DL, Straus S, Richardson WS, et al: Evidence-based medicine. How to practice and teach EBM. Edinburgh, Churchill Livingstone, 2000
72. Schwartz LH, Ferrand R, Boelle PY, et al: Lack of correlation between the location of choroidal melanoma and ultraviolet-radiation dose distribution. Radiat Res 147:451–6, 1997
73. Schwartz SM, Weiss NS: Place of birth and incidence of ocular melanoma in the United States. Int J Cancer 41:174–7, 1988
74. Scotto J, Fraumeni JF, Lee JA: Melanomas of the eye and other noncutaneous sites: epidemiologic aspects. J Natl Cancer Inst 56:489–91, 1976
75. Seddon JM, Gragoudas ES, Glynn RJ, et al: Host factors, UV radiation, and risk of uveal melanoma. A case-control study. Arch Ophthalmol 108:1274–80, 1990
76. Shammas HF, Watzke RC: Bilateral choroidal melanomas. Case report and incidence. Arch Ophthalmol 95:617–23, 1977
77. Singh AD, Topham A: Incidence of uveal melanoma in the United States: 1973–1997. Ophthalmology 110:956–61, 2003
78. Singh AD, Wang MX, Donoso LA, et al: Genetic aspects of uveal melanoma: a brief review. Semin Oncol 23:768–72, 1996
79. Sliney DH: Epidemiological studies of sunlight and cataract: the critical factor of ultraviolet exposure geometry. Ophthalmic Epidemiol 1:107–19, 1994
80. Strickland D, Lee JA: Melanomas of eye: stability of rates. Am J Epidemiol 113:700–2, 1981
81. Swerdlow AJ: Epidemiology of eye cancer in adults in England and Wales, 196–1977. Am J Epidemiol 118:294–300, 1983
82. Swerdlow AJ: Epidemiology of melanoma of the eye in the Oxford Region, 1952–78. Br J Cancer 47:311–3, 1983
83. Teikari JM, Raivio I: Incidence of choroidal malignant melanoma in Finland in the years 1973–1980. Acta Ophthalmol (Copenh) 63:661–5, 1985

Incidence of Uveal Melanoma in Sweden from 1960 to 1998

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Purpose. To investigate the incidence of uveal melanoma in Sweden during the period from 1960 to 1998, with respect to age distribution, gender, and changes in incidence over time.

Methods. The Swedish Cancer Registry was searched for patients with uveal melanoma and cross-checked against hospital files over patients where an eye-sparing treatment had been applied, to ensure inclusion in the Registry even when no histologic specimen was available. The crude and age-standardized incidence was estimated separately for each gender. The Swedish population of 1970 to 1974 was used as a standard, and the annual change in incidence was calculated by using a regression model with logarithmic incidence numbers.

Results. In total, 2997 patients met the criteria, of whom 1542 were males and 1455 females. During the 39-year period, the age-standardized incidence of uveal melanoma declined significantly in the male population, from 11.7 cases/million to 8.4 cases/million ($P = 0.002$). The trend toward reduced incidence in females, from 10.3 to 8.7 cases/million did not reach statistical significance ($P = 0.108$). The annual relative change in incidence was 1% (95% CI, 0.8%–1.2%) in males and 0.7% (95% CI, 0%–1.3%) in females. The age-specific incidence revealed a significantly higher incidence among men older than 45 years (23.5 cases/million) compared with the incidence in women of the same age group (19.2 cases/million; $P < 0.001$).

Conclusions. A Swedish national survey performed to establish the incidence of uveal melanoma during the period from 1960 to 1998 revealed a decreasing incidence in the male and a stable incidence in the female population. (*Invest Ophthalmol Vis Sci.* 2002;43:2579–2583)

The eye is the second most common site of malignant melanoma, (5.2%), as documented in a summary of more than 84,000 cases of melanoma from the U.S. National Cancer Data Base.[1] Uveal melanoma accounts for 85% of ocular melanomas and is by far the most common primary intraocular malignancy in the population older than 15 years.[2] The annual incidence in Scandinavia has previously been reported stable at approximately 5.0 to 7.5 cases/million inhabitants.[3–9] The incidence in populations with fair complexion and light iris color is likely to be high, because these features are considered strong risk factors for uveal melanoma.[10,11]

In Israel, where the phenotypes of Jewish immigrants from many countries are exceptionally heterogeneous, significant differences mark the incidence of uveal melanoma among the individual subpopulations. The highest incidence, 7.5 cases/million occurs in Jews of European-American origin, whereas the lowest incidence, 1.6 to 2.8 cases/million has been estimated in Jews born in Africa or Asia.[12] The incidence rate in Israelis was later reported to be stable and was no higher in descendants of high-risk subpopulations, despite their having been reared in an environment with more exposure to ultraviolet (UV) light.[13]

Because the incidence of cutaneous melanoma in the Swedish population has increased considerably during the past several decades, we wanted to investigate and clarify the incidence pattern of uveal melanoma in Sweden, based on data from a nationwide and population-based registry.

Materials and Methods

The Swedish National Cancer Registry, founded in 1958, receives reports when a malignant disease is diagnosed. Compulsory dual registration from the clinician and pathologist-cytologist ensures inclusion. Each patient is identified through a unique national registration number, and the registry is estimated to include more than 95% of all cases of cancer in the country.[14]

The research protocol for the present study was approved by the Human Ethics Committee at the Karolinska Institute, in accordance with the statutes of the World Medical Association's Declaration of Helsinki. Files from the Swedish Cancer Registry during the period from 1960 to 1998[15] were searched for patients with intraocular melanoma, using both the International Classifications of Diseases, Seventh Revision (ICD-7) code and the Systematized Nomenclature of Medicine (SNOMED) code. In the instances in which an eye-sparing treatment had been applied, the data files were double-checked against hospital files covering all patients treated with radioactive plaques, along with the records from a small group of patients who received proton beam irradiation. These treatment modalities were the only alternatives to local resection or enucleation during the investigation period. The eye-sparing treatments were centralized in two university clinics in Sweden.

The search of hospital files revealed 140 patients treated for uveal melanoma who were not included in the original files from the Cancer Registry. Therefore, the noninclusion rate was 4.7%. The missing cases were included in the database of the present study and reported to the Cancer Registry. The database thus revealed 2997 cases of uveal melanoma, including melanomas of the iris, ciliary body, and choroid.

Each gender was analyzed separately, and the patients were divided into eight age groups, each spanning 10 years, except the youngest (0–24 years) and the oldest (>85 years) age groups. The analysis over time was performed for 5-year periods from 1960, except the period from 1995 to 1998.

Files from the National Central Bureau of Statistics[16] including records from the Swedish population censuses of 1960 through 1998 and immigration statistics were used. Before 1970, the statistics were incomplete regarding the age distribution and gender of immigrants and so did not permit analysis for the period 1960 to 1969. The country of birth was not specified until 1980. Previously, only the original citizenship was noted in the files. For this study we accepted patients'

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Supported by a grant in memory of Ingrid Helén from Crown Princess Margaretha's Foundation for the Visually Impaired, Stockholm, Sweden, and by grants from the Stockholm Cancer Society, Sweden.

Submitted for publication November 19, 2001; revised March 11, 2002; accepted March 22, 2002.

Commercial relationships policy: N.

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TABLE 1. Stratum Weights of the Swedish Population during the Period 1970 to 1974

Age Groups (y)	Males	Females
0–24	0.36	0.34
25–34	0.15	0.14
35–44	0.11	0.11
45–54	0.13	0.13
55–64	0.12	0.12
65–74	0.08	0.10
75–84	0.04	0.05
>85	0.01	0.01

original citizenship as an estimate of birthplace when calculating the proportion of immigrants in the Swedish population before 1980.

An overall crude and age-standardized incidence rate in the total Swedish population was calculated, and the genders were further analyzed separately. Age-standardization on incidence numbers over the study period was performed by a direct method, with the Swedish population during the period 1970 to 1974 taken as a standard, according to the stratum weights shown in Table 1. The relative change in incidence over the 39-year period was calculated by linear regression after logarithmic transformation of incidence data. The annual change was expressed as a percentage with 95% confidence intervals. The level for statistical significance was set at a $P \leq 0.05$. The probabilities were corrected with the Bonferroni adjustment when subgroup comparisons were made.

The number of immigrants to Sweden born outside Europe was estimated for the years 1970, 1980, 1990, and 1998. In the calculations the extreme value of null incidence of uveal melanoma was set for this subpopulation. The influence of immigrants on the incidence of uveal melanoma for each gender was estimated by using the crude incidence numbers in the Swedish population of 1970, 1980, 1990, and 1998. These incidence numbers were then compared with the estimated incidence in the population when the immigrants were excluded. The crude incidence numbers in the Swedish population were expressed with 95% confidence intervals. Data were processed with statistical-analysis software (SPSS, ver. 10.0; SPSS Inc., Chicago, IL).

To compare the average density of uveal melanoma per ocular area unit in relationship with that of cutaneous melanoma the total uveal area of both eyes was estimated. According to the 0.5 mm shorter average axial length in females, the uveal area was estimated to be 34 cm^2 in males and 33 cm^2 in females.[17,18] The mean body surface area has previously been found to be 1.9 m^2 in men and 1.7 m^2 in women.[19] During the investigation period specified (1960–1998) 17,090 and 18,334 cases of cutaneous melanoma were diagnosed in the male and female Swedish population, respectively,[15] and the ratios of uveal versus cutaneous melanomas per surface unit consequently could therefore be calculated.

RESULTS

During the period 1960 to 1998 a total of 2997 patients with uveal melanoma was reported to the Swedish National Cancer Registry. Of these patients, 1542 were males and 1455 females (male-female ratio, 1.06). The males' ages ranged from 11 to 94 years, and the females' from 8 to 95 years. In the group younger than 25 years, there were 10 females and 8 males, but only three were younger than 15 years (two girls, aged 8 and 10 years, and a boy aged 11 years). Judging from the crude incidence shown in Figure 1, it appears that uveal melanoma was an extremely uncommon disease in the young population, with an age-specific incidence (Fig. 2) of 0.1 and 0.2 male and female cases/million, respectively, in the age group of less than 25 years. In the 25- to 34-year age group the incidence was low (1.7 cases/million for both genders), but in the population of 35- to 44-year-olds, the incidence increased to 4.0 and 5.6

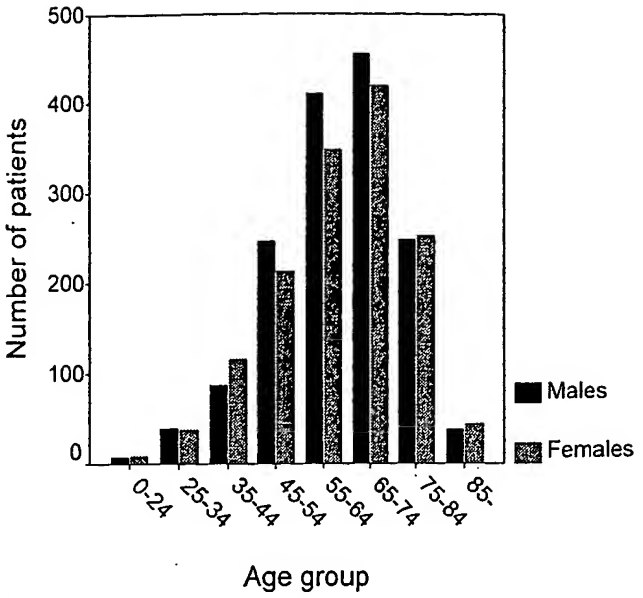

FIGURE 1. The age distribution of incidence of uveal melanoma in the male and female Swedish population during the period from 1960 to 1998, based on reports in the Swedish Cancer Registry.

cases/million, respectively. The age distribution revealed that the peak incidence appeared in a slightly earlier age group in females (65–74 years; 26.5 cases/million) compared with males, in which the maximum incidence (36.6 cases/ million) occurred in the 75- to 84-year age group. The difference in crude incidence between genders in the age groups older than 45 years was statistically significant, with the male incidence

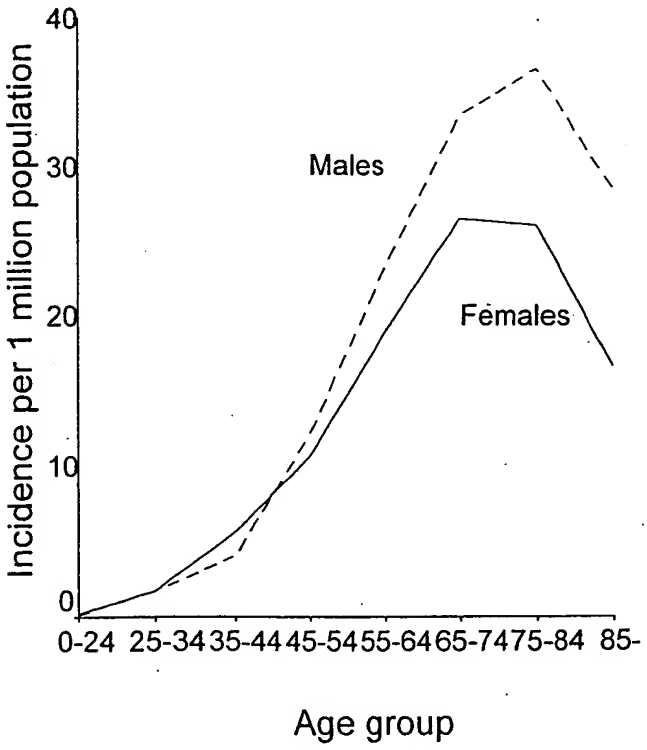

FIGURE 2. Age-specific incidence rates of uveal melanoma in the male and female populations of Sweden during the period from 1960 to 1998. In the age groups older than 45 years, a statistically significant male predominance was observed (23.5 cases/million) compared with the incidence among the females (19.2 cases/million; $P < 000.1$).

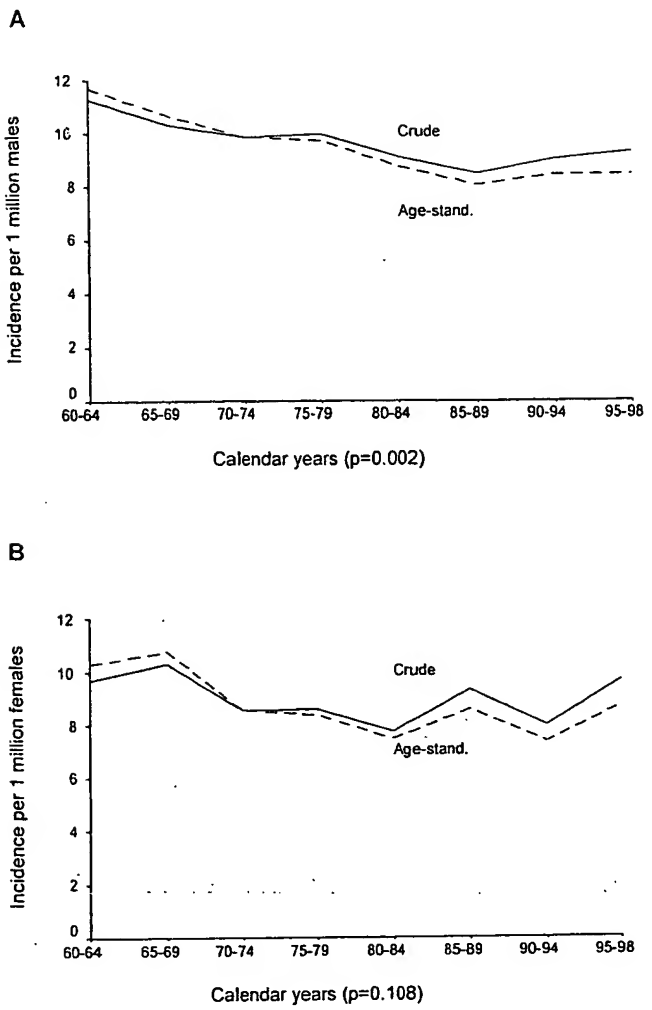

FIGURE 3. Age-standardized incidence rates of uveal melanoma in the Swedish (A) male and (B) female populations during the period from 1960 to 1998. *Solid line*: crude incidence; *broken line*: age-standardized rates when standardized against the Swedish population of 1970 to 1974. During the 39-year study period, the estimated incidence declined in males as from 11.7 to 8.4 cases/million ($P = 0.002$) with an annual relative change of 1%. In females the incidence rate was reduced from 10.3 to 8.7 cases/million at the end of the period, a decrease that was not statistically significant ($P = 0.108$). The annual relative change was 0.7% in females.

estimated at 23.5 cases/million inhabitants compared with the female incidence of 19.2 cases/million ($P < 0.001$), as illustrated in Figure 2.

During the period from 1960 to 1998 the age-standardized incidence of uveal melanoma in the total Swedish population was reduced from 11.0 cases/million to 8.5 cases/million ($P = 0.006$). In the male population, a significant reduction in incidence was found. The calculated age-standardized incidence decreased from 11.7 to 8.4 cases/million by the end of study period ($P = 0.002$). The female population also manifested a declining trend in incidence, from 10.3 to 8.7 cases/million during the investigation period, although the difference was not statistically significant ($P = 0.108$), as Figure 3 depicts. By applying a log regression model, we determined the annual change in incidence rate. The male incidence declined 1% yearly (95% CI, 0.8%–1.2%) whereas the female incidence decreased 0.7% (95% CI, 0%–1.3%) annually from 1960 to 1998.

The influence of non-European immigrants (with a presumably lower incidence of uveal melanoma) was analyzed for the years 1970, 1980, 1990, and 1998. For both genders, the estimated crude incidence for the population excluding the immigrants did not differ significantly from the true crude incidence, which included them (Table 2).

To evaluate whether the diagnostic criteria for uveal melanoma changed over the investigative period, one of the authors (Seregard S, unpublished data, 2001) reexamined a random sample of 916 paraffin-embedded specimens originally reported to be uveal melanoma during the period from 1960 to 1998. The specimens originated from several laboratories across Sweden. In only three cases (0.33%) did the original diagnosis require alteration, which confirms the high level of accuracy in the Cancer Registry.

The average tumor density (i.e., skin compared with uveal melanomas per unit of body area) of uveal melanomas was found to be 50 times higher in males and 41 times higher in females than that of cutaneous melanomas.

DISCUSSION

Throughout the 39-year investigation period, the incidence of uveal melanoma in Sweden was remarkably high from a global perspective. In the first period (1960–1964) the age-standardized incidence was 11.7 and 10.3 cases/million in males and females, respectively. The incidence declined to 8.1 and 8.6 cases/million during the period 1995 to 1998, a level similar to the incidence recently reported for Jews of Polish and Romanian ancestry.[13]

The incidence of uveal melanoma in Scandinavia was once recognized as among the highest worldwide. In Denmark, Jensen[3] reviewed the period from 1943 to 1952 and found a crude, but not age-standardized incidence of uveal melanoma of 7.4 cases/million. Østerlind[7] later analyzed the Danish population of 1943 to 1982 with age standardization and found a stable incidence of ocular melanoma (conjunctival melanomas

TABLE 2. Crude Incidence of Uveal Melanoma in the Swedish Male and Female Population Compared with the Estimated Incidence Excluding Non-European Immigrants

	Incidence*		Incidence Excluding Immigration*		95% CI†	
Year	Males	Females	Males	Females	Males	Females
1970	10.1	9.4	10.1	9.5	7.1-13.6	6.7-13.0
1980	9.5	8.2	9.7	8.3	6.7-13.0	5.6-11.4
1990	8.8	8.6	9.0	8.9	6.2-12.1	6.0-11.8
1998	9.3	9.7	9.7	10.1	6.7-12.7	7.0-13.0

Incidence in the non-European immigrants was set at 0.

* The incidence in cases/million.

† The estimated 95% confidence interval for the crude incidence in the total male compared with the female population is given.

included) of 7.5 and 6 cases/million in males and females, respectively, during that period. In the United States with its heterogeneous population, the age-standardized incidence of ocular melanoma was estimated at 6 cases/million, based on the Third National Cancer Survey during the period from 1969 to 1971.[20] Because different standard populations, and, hence, stratum weights, were used in these studies, the figures are not fully comparable. Other investigations, mainly in white populations have resulted in similar data.[21-23] However, in many earlier studies, the incidence of ocular malignancies in the population older than 15 years has been used as an approximation for the incidence of uveal melanoma, suggesting inaccuracies stemming from inclusion of too broad a group. The temporal trends in incidence have often been regarded as stable, although with small fluctuations in many populations.[5,13,24,25] A recent investigation in the United States (Singh A, personal communication, 2001) supports the impression of a stable incidence of uveal melanoma during the period 1973 to 1997, with an overall rate of 4.3 cases/million inhabitants.

However, in our data, we detected a statistically significant reduction in the incidence of uveal melanoma of 1% yearly for the male population in Sweden during the period from 1960 to 1998. In the female population, the incidence rates declined by 0.7% annually, but the reduction did not quite reach a statistically significant level ($P = 0.108$). A slight predominance of males has been found in several epidemiologic studies on uveal melanoma. To our knowledge no statistically significant age-dependent gender difference has previously been demonstrated for this tumor. However, we discovered a significantly higher incidence in males than in females in the group more than 45 years of age, although the reasons are unknown.

Until brachytherapy became a preferred treatment for smaller melanomas, almost every eye with uveal melanoma was enucleated and a specimen submitted for pathologic analysis. Ocular pathology in the earlier decades of the study period was not centralized, and therefore various pathologists at different laboratories examined the specimens. In an evaluation to determine whether the diagnostic criteria for uveal melanoma changed over the investigative period, reexamination of specimens identified misdiagnoses in only 0.33%. The diagnostic accuracy appears to be in the same low range as reported in the Collaborative Ocular Melanoma Study (COMS) in which the rate of misdiagnoses was 0.3%.[26] The risk of not reporting uveal melanoma cases could have increased in later years, because more eyes are treated without obtaining a pathology specimen, and therefore the cases were not reported dually by both clinician and pathologist. To reduce this risk, hospital files from the only two centers treating patients with eye-sparing techniques (brachytherapy and proton beam irradiation) were checked and missing patients included in the study.

Although the Swedish population was once considered ethnically homogeneous, a small but growing immigration from both European and non-European countries has occurred in the past few decades. However, the net immigration rate remains comparatively low: the Swedish rate is estimated at 0.86/1000 population compared with 3.5/1000 population for the United States during 2000.[27] Data from the Swedish National Bureau of Statistics[16] indicate that the proportion of the population of non-European origin in 1960 was 0.2% and in 1998 had increased to 4.0%.

Furthermore, the age distribution of the immigrant population differed, because it was proportionally younger than the total Swedish population for every period investigated. The influence on the crude incidence of uveal melanoma for each gender over time when taking into account a presumably lower incidence in the immigrant population was estimated and found to be insignificant. Therefore, immigration alone probably does not account for the decreasing incidence of uveal melanoma documented in this study.

The stable incidence rates of uveal melanoma published since the 1950s contrasts sharply with the well acknowledged increase in incidence of skin melanoma in populations with fair complexions.[28] Our results concur with those found in a recently published population-based Swedish survey of incidence rates for vulvar melanoma.[29] At this extracutaneous UV-light protected site, the incidence declined 3% annually during the investigation period of 1960 to1984.

The average density of cutaneous melanoma is lower than that of uveal melanoma when the relationship between the skin area and the uveal area is considered. Specifically, uveal melanomas are 50 times more common in men and 41 times more common in women than cutaneous melanomas. Also, vulvar melanomas were found to be overrepresented by a factor of 2.5, compared with cutaneous melanomas.[30]

The potential role of UV light in the development of uveal melanoma is a matter of controversy. Some studies indicate that sunlight exposure may be a risk factor,[10,31,32] but others found no clear latitudinal gradient.[20] Nearly all UV light in the adult eye is absorbed before reaching the uvea, although greater transmission is possible in childhood,[33] and a blue or light-colored iris is also suggested to transmit more UV-light.

Our results in a national survey in Sweden during a 39-year period point out a stable, and, in males, even decreasing, incidence of uveal melanoma during the same period that the incidence of cutaneous melanoma increased four to five times. The results therefore suggest that the initiation of uveal melanoma is less dependent on UV light exposure than that of cutaneous melanoma.

References

1. Chang AE, Hynds Karnell L, Menck HR. The national cancer database report on cutaneous and noncutaneous melanoma: a summary of 84,836 cases from the last decade. *Cancer.* 1998;83:1664-1678.
2. Hakulinen T, Teppo L, Saxén E. Cancer of the eye: a review of trends and differentials. *World Health Stat Q.* 1978;31:143-158.
3. Jensen OA. Malignant melanomas of the uvea in Denmark 1943-1952: a clinical, histopathological and prognostic study. *Acta Ophthalmol Suppl (Copenh).* 1963;75:53-78.
4. Ravio I. Uveal melanoma in Finland: an epidemiological, clinical, histological and prognostic study. *Acta Ophthalmol Suppl (Copenh).* 1977;133:5-26.
5. Teikari JM, Ravio I. Incidence of choroidal malignant melanoma in Finland in the years 1973-1980. *Acta Ophthalmol (Copenh).* 1985; 63:661-665.
6. Abrahamsson M. Malignant melanoma of the choroid and ciliary body 1956-1975 in Halland and Gothenburg: incidence, histopathology and prognosis. *Acta Ophthalmol (Copenh).* 1983;61:600-610.
7. Østerlind A. Trends in incidence of ocular malignant melanoma in Denmark 1942-1987. *Int J Cancer.* 1987;40:161-164.
8. Gislason I, Magnússon B, Tulinius H. Malignant melanoma of the uvea in Iceland 1955-1979. *Acta Ophthalmol. (Copenh).* 1985;63: 389-394.
9. Mork T. Malignant neoplasms of the eye in Norway: incidence, treatment and prognosis. *Acta Ophthalmol (Copenh).* 1961;39: 824-831.
10. Tucker MA, Shields JA, Hartge P, Augsburger J, Hoover RN, Fraumeni JF Jr. Sunlight exposure as risk factor for intraocular malignant melanoma. *N Engl J Med.* 1985;313:789-792.
11. Vajdic CM, Kricker A, Giblin M, et al. Eye color and cutaneous nevi predict risk of ocular melanoma in Australia. *Int J Cancer.* 2001; 92:906-912.
12. Iscovich J, Ackerman C, Andreev H, Pe'er J, Steinitz R. An epidemiological study of posterior uveal melanoma in Israel, 1961-1989. *Int J Cancer.* 1995;61:291-295.

3. Iscovich J, Abdulrazik M, Pe'er J. Posterior uveal malignant melanoma: temporal stability and ethnic variation in rates in Israel. *Anticancer Res.* 2001;21:1449-1454.
4. Mattson B, Wallgren A. Completeness of the Swedish Cancer Register: non-notified cancer cases recorded on death certificates in 1978. *Acta Radiol (Oncol).* 1984;23:305-313.
5. *The Cancer Registry: Cancer Incidence in Sweden.* Stockholm: National Board of Health and Welfare. Annual publications, 1960-1998.
6. *Official Statistics of Sweden.* Stockholm: The National Swedish Central Bureau of Statistics; Annual Publications. 1960-1998.
7. Duke-Elder S, Wybar K. The anatomy of the visual system. In: Duke-Elder S, ed. *System of Ophthalmology.* Vol 2. London: Henry Kimpton; 1961:80-81.
18. Ninn-Pedersen K, Stenevi U, Ehinger B. Cataract patients in a defined Swedish population 1986-1990: preoperative observations. *Acta Ophthalmol (Copenh).* 1994;72:10-15.
19. Swedish Survey of Living Conditions (ULF Investigation). In: *Official Statistics of Sweden.* Stockholm: The National Swedish Central Bureau of Statistics; 1999.
20. Scotto J, Fraumeni JF Jr, Lee JA. Melanomas of the eye and other noncutaneous sites: epidemiologic aspects. *J Natl Cancer Inst.* 1976;56:489-491.
21. Swerdlow AJ. Epidemiology of melanoma of the eye in the Oxford region 1952-78. *Br J Cancer.* 1983;47:311-313.
22. Birdsell JM, Gunther BK, Alastair Boyd T, Grace M, Jerry LM. Ocular melanoma: a population-based study. *Can J Ophthalmol.* 1980;15:9-12.
23. Foss AJ, Dolin PJ. Trends in eye cancer mortality among adults in the USA and England and Wales. *Br J Cancer.* 1996;74:1687-1689.
24. Strickland D, Lee JA. Melanomas of eye: stability of rates. *Am J Epidemiol.* 1981;113:700-702.
25. Swerdlow AJ. Epidemiology of eye cancer in adults in England and Wales, 1962-1977. *Am J Epidemiol.* 1983;118:294-300.
26. Colloborative Ocular Melanoma Study Group. Histopathologic characteristics of uveal melanoma in eyes enucleated from the Collaborative Ocular Melanoma Study. COMS report 6. *Am J Ophthalmol.* 1998;125:745-766.
27. Publications and Reports. *The World Fact Book 2000.* Washington, DC: Central Intelligence Agency; 2000. Available at http://www.odci.gov/cia/publications/factbook/indexgeo.html. Accessed September 8, 2001.
28. Thörn M, Bergström R, Adami HO, Ringborg U. Trends in incidence of malignant melanoma in Sweden, by anatomic site, 1960-1984. *Am J Epidemiol.* 1990;132:1066-1077.
29. Ragnarsson-Olding B, Johansson H, Rutqvist LE, Ringborg U. Malignant melanoma of the vulva and vagina: trends in incidence, age distribution, and long-term survival among 245 consecutive cases in Sweden 1960-1984. *Cancer.* 1993;71:1893-1897.
30. Ragnarsson-Olding B, Kanter-Lewensohn L, Lagerlöf B, Nilson B, Ringborg U. Malignant melanoma of the vulva in a nationwide, 25-year study of 219 Swedish females: clinical observations and histopathological features. *Cancer.* 1999;86:1293-1284.
31. Seddon JM, Gragoudas ES, Glynn RJ, Egan KM, Albert DM, Blitzer PH. Host factors, UV radiation, and risk of uveal melanoma. *Arch Ophthalmol.* 1990;108:1274-1280.
32. Li W, Judge H, Gragoudas ES, Seddon JM, Egan KM. Pattern of tumor initiation in choroidal melanoma. *Cancer Res.* 2000;60:3757-3760.
33. Zigman S. Effects on near ultraviolet radiation on the lens and retina. *Doc Ophthalmol.* 1983;55:375-391.

Mutational Analysis of Selected Genes in the TGFβ, Wnt, pRb, and p53 Pathways in Primary Uveal Melanoma

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PURPOSE. It is known that the pRb pathway cell-cycle inhibitor $p16^{INK4A}$ plays a significant role in cutaneous melanoma and that alteration of $p16^{INK4A}$, which resides within the 9p21-22 locus that also contains $p15^{INK4B}$ and $p14^{ARF}$, may occur in up to one third of uveal melanomas. The absence of TGFβ responsiveness noted in cultured uveal melanoma cells also suggests that the TGFβ pathway plays a role in the formation of this tumor. Therefore, mutational screening was performed in several key genes in tumor-suppressor pathways that are known to be altered in some uveal melanomas.

METHODS. Using denaturing high-performance liquid chromatography (DHPLC) analysis and DNA sequencing, a series of 67 uveal melanomas were screened for inactivating mutations in the TGFβ pathway members Smad4 and TGFβ receptor type 2 (TGFβR2), the downstream cell-cycle inhibitor $p15^{INK4B}$, and the cell-cycle inhibitors $p14^{ARF}$ and $p16^{INK4A}$. $p16^{INK4A}$ was also investigated for promoter hypermethylation. Mutational analysis was also performed on the Wnt pathway gene β-catenin, known to be mutated in approximately one quarter of cutaneous melanoma cell lines.

RESULTS. Polymorphisms in $p16^{INK4A}$ were detected in 3 of 50 samples, but no inactivating mutations were detected in any of the genes screened. Promoter hypermethylation of $p16^{INK4A}$ was detected in 5 of 55 tumors, and loss of heterozygosity of the $p16^{INK4A}$ locus was detected in 5 of 16 tumors.

CONCLUSIONS. Most primary uveal melanomas do not appear to contain somatic mutations in Smad4, TGFβR2, $p14^{ARF}$, $p15^{INK4B}$, $p16^{INK4A}$, or β-catenin. However, methylation of the $p16^{INK4A}$ promoter and loss of heterozygosity of the $p14^{ARF}$-$p16^{INK4A}$ locus occurs in some tumors. (*Invest Ophthalmol Vis Sci.* 2002;43:2845–2851)

Uveal melanoma is the most frequently occurring primary intraocular tumor in white adults, having an annual incidence rate of 0.7 per 100,000 persons.[1] The eye is the commonest site for noncutaneous melanomas, accounting for approximately 80% of such lesions[2] and accounting for 13% of all deaths caused by melanoma, which has a very high mortality rate.[3] This tumor carries up to a 50% 5-year mortality,[4] depending on the tumor size.

Both uveal and cutaneous melanoma originate from the melanocyte, but little is known about the underlying molecular pathogenesis of uveal melanoma, in contrast to cutaneous melanoma, for which there have been more substantial advances in detecting mutations.[5] Both tumors differ significantly in their origins, with UV light appearing to play little or no part in the causation of uveal melanoma, unlike cutaneous melanoma.[6] Unlike cutaneous melanoma, no genes or tumor-suppressor pathways have so far been convincingly linked to uveal melanoma.[7] Uveal melanoma spreads hematogenously, leading to liver metastasis, whereas cutaneous melanoma spreads mainly through the lymphatics,[8] with skin metastases a more common problem. Various chromosomal abnormalities have been noted in uveal melanoma—predominantly, nonrandom alterations in chromosomes 3, 6, and 8. This is in contrast to cutaneous melanoma, which has only chromosome 6 alterations in common with uveal melanoma, and with further common cytogenetic changes in chromosomes 1, 7, 9, and 10.[5]

Transforming growth factor (TGF)-β is known to suppress the growth of normal human melanocytes, but this response is lost by approximately two thirds of ocular melanoma cells.[9] This loss of TGFβ responsiveness suggests that an abnormality of the TGFβ pathway or of downstream components could be a cause of formation of these tumors. In the adult eye, TGFβ is produced by the ciliary body and retina and mediates a variety of effects, including immune suppression and modulation of fibrosis in response to injury.[10] Secreted TGFβ binds to two different types of serine/threonine kinase receptors, known as TGFβ type 1 and type 2 (TGFβR1 and TGFβR2; for a review see Ref. 11). TGFβ binding activates various biochemical pathways through intracellular signaling molecules known as Smads. TGFβ stimulation is known to upregulate a number of cell-cycle inhibitors, including $p15^{INK4B}$ and $p27^{KIP1}$.

The cell cycle inhibitor $p15^{INK4B}$ is known to be part of the pRb tumor-suppressor pathway that regulates the G_1-S checkpoint of the cell cycle and is one of the most often deregulated pathways in cancer. $p16^{INK4A}$ is the main target for inactivation in this pathway by germ line mutation[12] and by promoter hypermethylation[13] in some cutaneous melanomas and in other cancers. The 9p21 INK4A-ARF locus that contains the $p16^{INK4A}$ gene undergoes allelic loss in between one quarter to one third of cutaneous[14] and uveal[15] melanoma tumors. The INK4A-ARF locus contains two tumor-suppressor genes, $p16^{INK4A}$ and $p14^{ARF}$ (lying 20 kb apart), which have different first exons (1α and 1β, respectively) and share exons 2 and 3, although $p14^{ARF}$ uses an alternative reading frame, so that the two proteins share no amino acid sequence homology.[16] $p14^{ARF}$ is another cell regulator protein, but acts through the p53 pathway rather than the pRb pathway.[17]

In addition, $p15^{INK4B}$ maps in the 9p21-22 region. This protein having a very similar if not identical function in the cell cycle by inhibiting cyclin dependent-kinase(CDK)-4, which phosphorylates and inactivates pRb protein.[18] Because of the proximity of the two genes, the high number of 9p21 alterations in many tumors can often overlap both genes. The

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Supported by Moorfields Eye Hospital Locally Organised Research Scheme and the Joint Research Board, St. Bartholomew's Hospital, London, United Kingdom.

Submitted for publication October 25, 2001; revised May 16, 2002; accepted May 29, 2002.

Commercial relationships policy: N.

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$p15^{INK4B}$ gene is known to be deleted along with $p16^{INK4A}$ in nearly 10% of cutaneous melanoma tumors.[19] $p15^{INK4B}$ mutations have been found in a number of cancers, particularly leukemias (up to 30% of some forms).[18] Rare point mutations of the $p14^{ARF}$ (see Ref. 20) and $p15^{INK4B}$ (see Ref. 19) genes have been detected in a few cases of cutaneous melanoma tumors.

The Wnt/wingless pathway gene β-catenin was also screened for mutations, because it has been shown to be mutated in approximately one quarter of cutaneous melanoma cell lines.[21] The Wnt pathway is mediated by β-catenin, which activates members of the LEF1/TCF transcription factor family—in turn, activating the transcription of Wnt target genes such as cyclin D1.

Herein, we describe analysis of key members of the TGFβ, pRb, and Wnt pathways for somatic mutations in sporadic uveal melanoma. We specifically examined key components of these pathways that have previously been shown to be mutated or methylated in cutaneous melanoma and also other cancers. Mutational analysis was confined to exons that contain known mutational hot spots. Denaturing high-performance liquid chromatography (DHPLC; Transgenomic, Crewe, UK) was used to look for mutations, because of its high throughput and sensitivity. Genes screened include the TGFβ pathway member TGFβR2 (exons 3, 5, and 7), the mutation of which is known to play a role in the formation of colon and gastric cancers.[22] Another TGFβ pathway member investigated was Smad4 (exons 8–11), because it is known to be mutated in pancreatic cancers.[23] The exon 3 region of the β-catenin gene is known to be mutated in numerous cancers and was sequenced in many of our tumors. Allelic loss of the 9p21-22 $p16^{INK4A}$ locus has been observed in approximately 25% of both uveal[15] and cutaneous[24] melanoma tumors, and we therefore decided to look at this region in our series of tumors (Fig. 1). We also screened for mutations in the cell-cycle regulatory genes mapping to the 9p21 locus: $p16^{INK4A}$ (exons 1α and 2), $p14^{ARF}$ (exon 1β), and $p15^{INK4B}$ (exon 2).

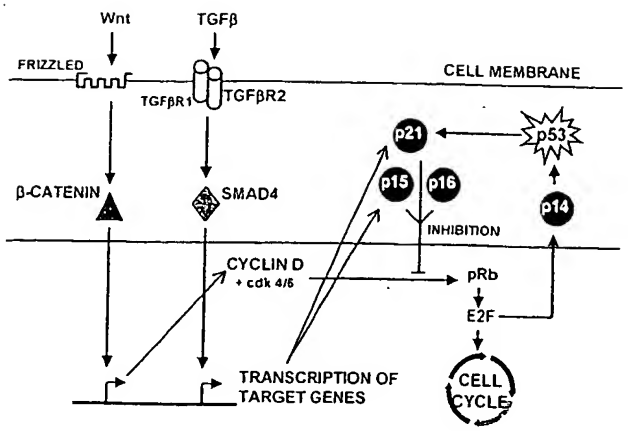

FIGURE 1. The Wnt, p53, TGFβ, and pRb pathways, and their role in the cell cycle. Key members of these pathways that were screened for mutations are shown. Wnt ligands activate membrane-bound Frizzled receptors and stop the degradation of β-catenin. Nuclear β-catenin then can activate Wnt target genes, such as cyclin D1. In TGFβ signaling, TGFβ activates membrane bound TGFβ receptors 1 and 2 to propagate a signal through the Smad family of proteins. A Smad complex including Smad4 activates transcription of genes including p15 and p21. In the pRb pathway, pRb protein is phosphorylated by a cyclin D-CDK complex to activate E2F, which activates the expression of genes essential for G_1–S phase progression of the cell cycle. The phosphorylation of pRb can be inhibited by CDK inhibitors p21, p16, and p15. E2F also induces $p14^{ARF}$, which sequesters MDM2, thereby preventing p53 degradation. p53 in turn activates p21, which induces apoptosis and performs many other functions.

MATERIALS AND METHODS

Melanomas

A total of 66 primary uveal melanomas collected from enucleations at Moorfields eye hospital were used in this study, along with a uveal melanoma liver metastasis. The median age of the patients was 61 years, and there was a small bias toward male specimens (56%). These tumors were predominantly choroidal (74% of tumors), with smaller numbers of ciliary body (14%) and mixed choroidal–ciliary body (12%) type. They were also mostly of spindle cell-type (41%), with a slightly lower number of mixed (32%) and epithelioid (27%) types. All tumor samples were removed as part of the patient's treatment and with local ethics committee approval for use of the tissue in this study. The study protocol adhered to the tenets of the Declaration of Helsinki. For transport to the laboratory, the enucleated eyes were placed in sterile DMEM (Sigma, Poole, UK) with penicillin and streptomycin. After careful macroscopic examination for possible extrascleral extension and transillumination, the eye was cut vertically or horizontally through the tumor mass to one side of the optic nerve with a dermatome blade. Part of the tumor present in the calotte was snap frozen in liquid nitrogen for DNA extraction and subsequent molecular studies.[9] DNA extraction was performed with a kit (Nucleon; Tepnel Life Sciences, Manchester, UK).

Polymerase Chain Reaction

PCR reactions were of 50-μL volume, with a commercial PCR buffer (Bioline, London, UK), 1 mM $MgCl_2$, 200 μM dNTP, 20 to 100 ng genomic DNA template, and 0.5 U *Taq* polymerase, unless otherwise specified. All PCR assays were run on a commercial system (Hybaid, Ashford, UK). PCR primer sequences were taken from previously published reports (see Table 1 for references for all exons screened) or designed from genomic sequences to exons of interest. These primer pairs either spanned the entire exon and intron–exon junction or were split into two overlapping PCR products if the fragments were too long for DHPLC analysis and sequencing. Exons that were too large and had to be split were $p16^{INK4A}$ exon 2 and $p14^{ARF}$ exon 1β. PCR products were then used for mutational analysis by DHPLC. Exon 3 of β-catenin did not produce a PCR fragment suitable for DHPLC analysis, and PCR was therefore undertaken on 10 tumors and the products sequenced. β-Catenin exon 3 and $p15^{INK4B}$ exon 2 were not covered fully by these PCR fragments, but the exon 3 fragment had 88.5% of the exon sequence including all the key glycogen synthase kinase-3β phosphorylation site,[25] and the $p15^{INK4B}$ exon 2 fragment represented more than 80% of the exon sequence. The $p15^{INK4B}$ exon 2 primers were a gift from Nadem Z. Soufir (Laboratoire de Biochimie B, Hormonologie et Genetique, Hopital Bichat-Claude-Bernard, Paris).

Heteroduplex Formation and DHPLC Analysis

Heteroduplex formation was performed by heating the PCR products for 5 minutes at 94°C, followed by cooling to 40°C at a rate of 0.03°C per second, and the PCR products were then analyzed by DHPLC. The optimum temperatures for the analysis for each fragment were calculated by computer (WAVE-Maker software; Transgenomic). Any variants detected were then purified and sequenced.[30] In addition, for each exon screened PCR products for 10 tumors were also sequenced to confirm the data generated by DHPLC.

PCR Product Purification and Sequence Analysis

PCR products were purified, using a PCR purification kit (QIAquick; Qiagen, Crawley, UK). Purified PCR products are then directly sequenced (Big-Dye terminator chemistry; Applied Biosystems, Warrington, UK) and analyzed (model 377 automated sequencer; Applied Biosystems).

TABLE 1. Primer Sequences Used, with References

Gene	Exon	Primer Sequence	Annealing Temperature	Reference
Polymerase chain reaction				
TGFβR2	3	F: TGCAATGAATCTCTTCACTC R: CCCACACCCTTAAGAGAAGA	55°C	22
	5	F: GGCAGCTGGAATTAAATGATGGGC R: TGCTCGAAGCAACACATG	55°C	22
	7	F: CCAACTCATGGTGTCCCTTTG R: TCTTTGGACATGCCCAGCCTG	55°C	22
Smad4	8	F: TGTTTTGGGTGCATTACATTTC R: CAATTTTTTAAAGTAACTATCTGAC	54°C	26
	9	F: TATTAAGCATGCTATACAATCTG R: CTTCCACCCAGATTTCAATTC	54°C	26
	10	F: AGGCATTGGTTTTTAATGTATG R: CTGCTCAAAGAAACTAATCAAC	57°C	26
	11	F: CAAAAAGTGTGCAGCTTGTTG R: CAGTTTCTGTCTGCTAGGAG	57°C	26
$p14^{ARF}$	1β—1	F: TCAGGGAAGGGCGGGTGCG R: GCCGCGGGATGTGAACCA	60°C	16
	1β—2	F: GCCGCGAGTGAGGGTTTT R: CACCGCGGTTATCTCCTC	60°C	16
$p15^{INK4B}$	2	F: GGCTCTGACTCTGCT R: GTGGGCGGCTGGGGAACC	64°C	Gift from N. Soufir
$p16^{INK4A}$	1	F: GGGAGCGCATGGAGCCG R: AGTCGCCCGCCATCCCCT	63°C	27
	2A	F: AGCTTCCTTTCCGTCATGC R: GCAGCACCACCAGCGTG	55°C	27
	2—2	F: GACCCCGCCACTCTCACC R: GTGCTGGAAAATGAATGCTCTG	55°C	28
β-Catenin	3	F: ATGGAACCAGACAGAAAAGC R: GCTACTTGTTCTGAGTGAAG	56°C	25
Nested methylation PCR (inner and outer primer pairs)				
p16A (outer)	—	F: GGTTGGTTGGTTATTAGAGGGTGG R: TACAAACCCTCTACCCACCTAAAAT	56°C	29
p16B (inner)	—	F: GTTGGTTATTAGAGGGTGGGG R: CTCCIACCITAACTATTCIATAC	64°C	29

F, forward primer; R, reverse primer.

Methylation Analysis

Approximately 100 to 200 ng DNA from each tumor was sodium bisulfite modified. The DNA was denatured in 0.3 M NaOH in a volume of 20 μL for 15 minutes at 37°C. A solution of 120 μL 3.6 M sodium bisulfite and 0.6 mM hydroquinone was added, and the samples were cycled for 5 hours (30 seconds at 95°C, 15 minutes at 50°C for 40 cycles) in a PCR apparatus (Hybaid). The DNA was then desalted with a DNA purification resin (Wizard; Promega, Southampton, UK) and desulfonated by the addition of 5 M NaOH to 0.3 M and incubated at room temperature for 5 minutes. The DNA was neutralized with glacial acetic acid and then precipitated with two volumes of ethanol in the presence of yeast transfer (t)RNA and 0.01 M $MgCl_2$. DNA was then resuspended in 30 to 40 μL sterile water and amplified by nested PCR with *Taq* polymerase (Red-Hot *Taq*; ABgene, Epsom, UK) and buffers.[29]

Loss-of-Heterozygosity Analysis and Microsatellite Markers

Dye-labeled PCRs of highly polymorphic microsatellite markers positioned around the 9p21-22 $p16^{INK4A}$ locus (*D9S1748* and *D9S1749*) were used for loss-of-heterozygosity (LOH) analysis. This region is known to show allelic loss in numerous cancers, particularly in cutaneous melanoma. The reported heterozygosity of these markers was 0.87 and 0.94 for *D9S1748* and *D9S1749*, respectively. PCR was performed under standard cycling conditions on 18 extracted blood and tumor pairs. Microsatellite profiles were then visualized and analyzed in an automated sequencer (Li-Cor 4200 GeneReadIR; MWG, Milton Keynes, UK). The data were collected automatically and analyzed on computer (GeneImageIR RFLPscan Plus ver. 3.0; Scanalytics, Fairfax, VA).

LOH was quantitatively assessed according to the method of Okabe et al.[31] This method calculates the LOH index, defined as the allele ratio in normal tissue divided by the allele ratio of the tumor tissue. The allele ratio was calculated as the peak height of the smaller allele divided by the peak height of the larger allele. Allelic loss was defined by an LOH index of less than 0.5 or more than 2.0.

RESULTS

Mutational Analysis

No mutations were identified in any of our tumor samples in any of the exons screened. Screening of $p16^{INK4A}$ exon 2 produced variant DHPLC profiles (distinct doublet peaks) in three samples, with the sequencing showing that they were all heterozygous for a previously reported Ala148Thr polymorphism[32] (Fig. 2).

LOH Analysis

This series of tumors had previously undergone LOH analysis for the 3p22 TGFβR2 locus, finding allelic loss in approximately 30% of the tumors.[9] There was then enough DNA remaining in 18 blood and tumor pairs to allow for LOH analyses to be performed on a few more loci (Table 2, Fig. 3).

Of these 18 pairs of samples analyzed for 9p21-22 $p16^{INK4A}$ LOH, 2 (11.1%) samples were uninformative at both loci, 5 (27.8%) were informative for both loci , and 11 (61.1%) were

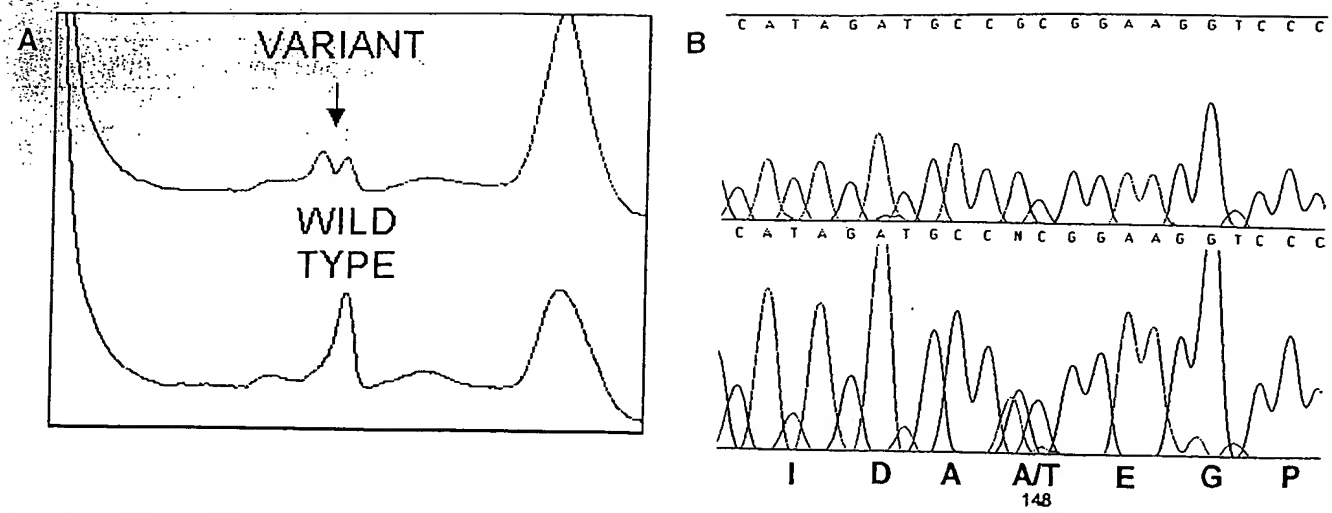

FIGURE 2. (A) A positive DHPLC trace, showing a wild-type sequence with a wild-type DHPLC profile and a $p16^{INK4A}$ exon 2 variant with a variant DHPLC profile and a distinct doublet peak. (B) Sequence showing the $p16^{INK4A}$ exon 2Ala148Thr polymorphism, with the wild-type sequence shown above the tumor's heterozygous sequence.

informative at only one locus. Of the 16 samples that were informative, 5 showed LOH in at least one of the loci (31%). Of these samples showing LOH, two displayed LOH at one locus only. Some samples could not be analyzed for technical reasons (labeled in Table 2 as ND, no data). After repeating analyses of most of these samples, we did not have enough DNA for further loci to be examined.

Methylation Analysis

Evidence of methylation of the $p16^{INK4A}$ promoter was detected in 5 (9.1%) of 55 of the tumors examined. Only the reverse-sequenced strand produced good enough quality sequencing (Fig. 4). Because of the small number of samples screened for LOH, it was not possible to determine whether there was any association between the samples that had $p16^{INK4A}$ methylation and 9p21-22 $p16^{INK4A}$ LOH.

DISCUSSION

In a previous study,[9] we examined the expression of members of the TGFβ pathway by immunocytochemistry, and found that the expression of Smads 2, 3, and 4; p27; and TGFβR2 was absent in a large percentage of uveal melanoma tumors, along with a loss of TGFβ responsiveness. In the current study, we investigated whether this loss of expression was due to somatic mutation of key members of these pathways—events that would be consistent with the absence of TGFβ responsiveness and with the potential abrogation of this and other pathways. Because uveal melanoma tumors have no obvious major inherited component[2] we concentrated on finding somatic mutations in these pathways. We also screened for genetic alterations several genes known to be altered in cutaneous melanoma—such as $p14^{ARF}$, $p15^{INK4B}$, $p16^{INK4A}$, and

TABLE 2. Table of LOH and $p16^{INK4A}$ Methylation Results for 18 Blood-Tumor Pairs

No.	Sample	Age at Enucleation	Site of Tumor	Cell Type	p16 Methylated	P16 LoH: 9p21-22	
						D9S1748	D9S1749
1	96/3	40	Choroid	Mixed	ND	NI	−
2	96/5	73	Choroid	Epithelioid	No	NI	−
3	96/6	39	CB/Choroid	Spindle	No	NI	ND
4	96/7	79	CB	Mixed	No	+	NI
5	96/8	42	Choroid	Spindle	No	−	NI
6	96/12	77	Choroid	Mixed	No	−	−
7	96/14	68	Choroid	Epitheloid	No	NI	−
8	96/16	48	Choroid	Spindle	No	+	−
9	96/17	67	Choroid	Spindle	Yes	−	−
10	96/18	64	Choroid	Spindle	No	+	ND
11	97/1	33	Choroid	Epitheloid	ND	NI	NI
12	97/5	54	Choroid	Spindle	No	−	−
13	97/6	36	Choroid	Spindle	No	−	ND
14	97/7	64	Choroid	Spindle	Yes	−	NI
15	97/8	85	Choroid	Spindle	ND	NI	−
16	97/10	31	CB	Epitheloid	No	−	+
17	97/11	62	Choroid	Spindle	No	NI	+
18	97/16	73	Choroid	Mixed	No	−	ND
A*	96/10	79	Choroid	Epitheloid	Yes	NT	NT
B*	96/15	63	Choroid	Mixed	Yes	NT	NT
C*	98/35	48	Choroid	Spindle	Yes	NT	NT

ND, no data; NT, not tested; NI, not informative; +, LOH; −, no LOH; CB, ciliary body.
*Tumors were shown to be methylated, but were not tested for LOH.

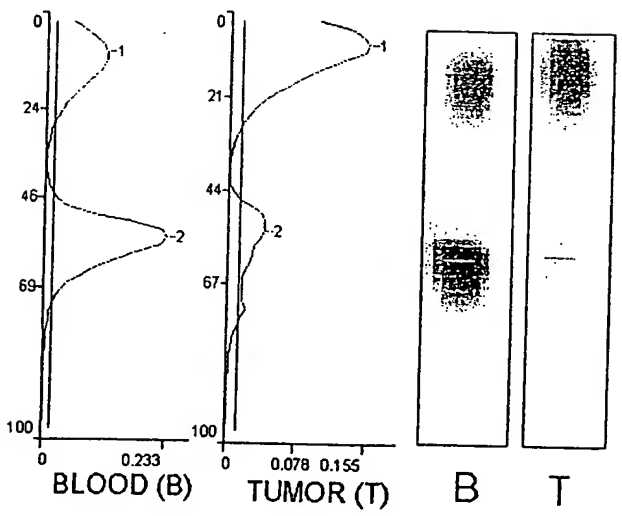

FIGURE 3. Electropherogram, showing LOH assessment on the chromosome 9p21-22 locus *D9S1749*. Blood is compared with tumor tissue, and the density units of each allele are compared (*x*-axis). The lower (*smaller*) allele is deleted in the tumor.

β-catenin—and we found it unlikely that mutation of these important members of the pRb, TGFβ, and Wnt pathways are responsible for disregulation in these pathways. It is known that DHPLC analysis has difficulty in resolving C-to-G transversions and also has difficulty analyzing high-melting domains embedded in low-melting DNA regions, such as β-catenin exon 3. Apart from these slight problems, DHPLC analysis is considered to be very accurate, with a sensitivity and specificity thought to be higher than 96%.[33] In a study screening 113 amplicons containing 14 different *BRCA1* mutations DHPLC resolved 100% of the alterations, compared with 96 using single-strand conformation polymorphism (SSCP) analysis.[34] Most mutations that were not detected by DHPLC would have been expected to be detected from the sequencing of each exon, especially in that we concentrated on codons with published mutations. Not all the exons were screened, and very little of the surrounding intronic sequence was examined; therefore, some mutations could have been missed by this analysis.

There were other genes in these pathways that have had mutations noted in certain cancers, Smad2 being a notable example, but many of these published mutations are thought to be very rare.[35] There is also a possibility the mutations are present in unscreened exons of these genes (e.g., Smad4 exons 1–7, $p16^{INK4A}$ exon 3). However, these regions do not have the same degree of structural conservation across the species and are without the same frequency of published mutations in other cancers as the mutational hot spots analyzed in this study.

A minority of these uveal melanoma tumors displayed methylation of $p16^{INK4A}$ promoter (10%) and allelic loss of the 9p21 $p16^{INK4A}$ locus (31%), although not in the same tumors. Because of the small number of samples and particularly the small number of positive samples, no significant correlations could be made between these DNA abnormalities and any clinical or histologic features. Ideally, we would have liked to examine further loci; but, unfortunately, DNA was limited. The level of 9p21 LOH observed in this study (31%) was consistent with previous molecular studies of uveal melanoma, which have found levels of $p16^{INK4A}$ LOH of between 24% and 32%.[36,15] Other studies have also detected $p16^{INK4A}$ homozygous deletions in approximately 12% of tumors[15] and intronic $p16^{INK4A}$ mutations.[37] The alterations noted may all contribute to lower levels of expression of many of these genes, and if there are enough key members of these pathways with reduced expression, these may act as contributory factors to the development of melanoma.

Previous studies have found constitutional $p16^{INK4A}$ mutations in a substantial proportion of familial cutaneous melanoma (>40%)[28] and in up to 75% of cutaneous melanoma cell lines,[38] but not in the small number of families predisposed to uveal melanoma,[16] although it is probable that most familial cases of uveal melanoma represent an aggregation of sporadic cases rather than true Mendelian inheritance. The number of sporadic cutaneous melanoma cases involving the $p16^{INK4A}$ mutation is much lower, varying between 3.3%[24] and 25%.[39] The frequency of $p16^{INK4A}$ methylation found (10%) is consistent with levels in cutaneous melanoma (10%)[13] and is between levels recorded in previous studies in uveal melanoma (6%[15] to 32%[40]). The higher frequency recorded by van der Velden et al.[40] was obtained by using the more sensitive technique of methylation-specific PCR, which can detect tumors with only partial methylation.

Samples were regarded as methylated when the level of each methylated CG dinucleotide was greater than the unmethylated dinucleotide (TG), suggesting greater than 50% methylation of the target DNA. There were a small number of samples that were unmethylated but had very low levels of CG (<5%), but we could not be sure whether this was due to methylation or to background unincorporated dye terminators in the sequencing trace. Low levels of methylation of this magnitude and even levels too small to be seen in the sequence trace are theoretically detected by the alternative technique used by van der Velden et al.[40] Because of the purity of the uveal melanoma tumor DNA samples, the lower levels of methylation noted are unlikely to be due to contamination from normal tissues. Contamination from partially bisulfite-modified DNA is also unlikely, because the primers were designed so that unmodified DNA would not amplify.

The absence of expression of Smads 2, 3, and 4; p27; and TGFβR2 noted in a large percentage of uveal melanoma tumors[9] without any obvious inactivating mutations discovered, could be explained by promoter hypermethylation of these genes. However Smad4, p27, and TGFβR2, have been screened for promoter hypermethylation in a small number of tumor

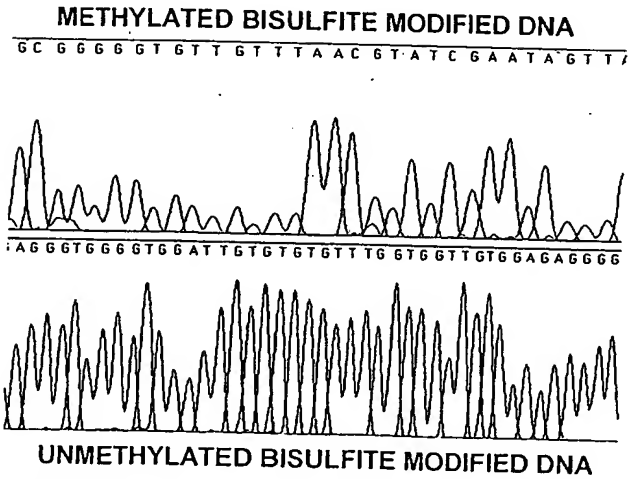

FIGURE 4. Examples of bisulfite-modified DNA sequence from uveal melanoma tumors showing methylated and unmethylated sequence of the $p16^{INK4A}$ promoter. In the unmethylated sequence, all cytosines are converted by the bisulfite modification step and PCR to thymidine, whereas, in the methylated sequence, all 5-methylcytosines are resistant to modification and remain cytosines. This DNA was extracted from a nonpigmented, spindle-cell choroidal tumor from a 48-year-old woman (sample 98/35, Table 2).

types, with no Smad4 methylation discovered to date.[41] Some promoter hypermethylation has been detected in a small number of cancer cell lines in the p27[42] and TGFβR2 genes,[43] although not in any significant amounts in primary tumors.[44,45] That there were no significant amounts of methylation of these genes in other tumors does not discount it as a plausible mechanism in uveal melanoma. This should be investigated further.

Uveal melanoma has a low cellular proliferation rate and is different than cutaneous melanoma in behavior and response to chemotherapy.[45] These findings further highlight the molecular differences between uveal and cutaneous melanoma and suggests that in uveal melanoma, methylation may play a more important role than somatic mutation.

In summary, no genetic mutations were detected in any of the Wnt, pRb, and TGFβ pathway genes that we screened, although in this series approximately 10% of the tumors had high levels of $p16^{INK4A}$ promoter methylation and approximately 31% of the tumors also showed loss of heterozygosity of the 9p21 $p16^{INK4A}$ locus. Because of the limited availability of samples, protein expression studies of could not be performed. Thus, further studies are needed to relate the molecular alterations in the 9p21 $p16^{INK4A}$ locus with $p16^{INK4A}$ protein expression. Mutations in Smad4, TGFβR2, β-catenin, $p16^{INK4A}$, $p15^{INK4B}$, and $p14^{ARF}$ are unlikely to be frequent contributors to uveal melanoma. So far, non-cancer-causing genes have been found to undergo inactivating mutations in most uveal melanomas, suggesting that there may be other mechanisms of tumorigenesis in uveal melanoma that are yet to be discovered.

Acknowledgments

The authors thank Veronique Bataille and Anthony G. Quinn for assistance in setting up the project and Vicky Brown, Nadem Zenedine Soufir, and Mohammed Ikram for technical assistance.

References

1. Prescher G, Bornfeld N, Hirche H, Horsthemke B, Jockel KH, Becher R. Prognostic implications of monosomy 3 in uveal melanoma. *Lancet.* 1996;347:1222-1225.
2. Sisley K, Parsons MA, Garnham J, et al. Association of specific chromosome alterations with tumour phenotype in posterior uveal melanoma. *Br J Cancer.* 2000;82:330-338.
3. Albert DM, Niffenegger AS, Willson JK. Treatment of metastatic uveal melanoma: review and recommendations. *Surv Ophthalmol.* 1992;36:429-438.
4. Foss AJ, Cree IA, Dolin PJ, Hungerford JL. Modelling uveal melanoma. *Br J Ophthalmol.* 1999;83:588-594.
5. Chin L, Merlino G, DePinho RA. Malignant melanoma: modern black plague and genetic black box. *Genes Dev.* 1998;12:3467-3481.
6. Dolin PJ, Foss AJ, Hungerford JL. Uveal melanoma: is solar ultraviolet radiation a risk factor? *Ophthalmic Epidemiol.* 1994;1:27-30.
7. Brantley MA Jr, Harbour JW. Inactivation of retinoblastoma protein in uveal melanoma by phosphorylation of sites in the COOH-terminal region. *Cancer Res.* 2000;60:4320-4323.
8. Seftor RE, Seftor EA, Hendrix MJ. Molecular role(s) for integrins in human melanoma invasion. *Cancer Metastasis Rev.* 1999;18:359-375.
9. Myatt N, Aristodemou P, Neale MH, et al. Abnormalities of the transforming growth factor-beta pathway in ocular melanoma. *J Pathol.* 2000;192:511-518.
10. Joyce NC, Zieske JD. Transforming growth factor-beta receptor expression in human cornea. *Invest Ophthalmol Vis Sci.* 1997;38:1922-1928.
11. Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature.* 1997;390:465-471.
12. Castellano M, Parmiani G. Genes involved in melanoma: an overview of INK4a and other loci. *Melanoma Res.* 1999;9:421-432.
13. Gonzalgo ML, Bender CM, You EH, et al. Low frequency of p16/CDKN2A methylation in sporadic melanoma: comparative approaches for methylation analysis of primary tumors. *Cancer Res.* 1997;57:5336-5347.
14. Okamoto A, Demetrick DJ, Spillare EA, et al. Mutations and altered expression of p16INK4 in human cancer. *Proc Natl Acad Sci USA* 1994;91:11045-11049.
15. Merbs SL, Sidransky D. Analysis of p16 (CDKN2/MTS-1/INK4A) alterations in primary sporadic uveal melanoma. *Invest Ophthalmol Vis Sci.* 1999;40:779-783.
16. Soufir N, Bressac-de Paillerets B, Desjardins L, et al. Individuals with presumably hereditary uveal melanoma do not harbour germline mutations in the coding regions of either the P16INK4A, P14ARF or cdk4 genes. *Br J Cancer.* 2000;82:818-822.
17. Stott FJ, Bates S, James MC, et al. The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *EMBO J.* 1998;17:5001-5014.
18. Drexler HG. Review of alterations of the cyclin-dependent kinase inhibitor INK4 family genes p15, p16, p18 and p19 in human leukemia-lymphoma cells. *Leukemia.* 1998;12:845-859.
19. Matsumura Y, Nishigori C, Yagi T, Imamura S, Takebe H. Mutations of p16 and p15 tumor suppressor genes and replication errors contribute independently to the pathogenesis of sporadic malignant melanoma. *Arch Dermatol Res.* 1998;290:175-180.
20. Rizos H, Puig S, Badenas C, et al. A melanoma-associated germline mutation in exon 1beta inactivates p14ARF. *Oncogene.* 2001;20:5543-5547.
21. Rimm DL, Caca K, Hu G, Harrison FB, Fearon ER. Frequent nuclear/cytoplasmic localization of beta-catenin without exon 3 mutations in malignant melanoma. *Am J Pathol.* 1999;154:325-329.
22. Vincent F, Nagashima M, Takenoshita S, et al. Mutation analysis of the transforming growth factor-beta type II receptor in human cell lines resistant to growth inhibition by transforming growth factor-beta. *Oncogene.* 1997;15:117-122.
23. Schutte M, Hruban RH, Hedrick L, et al. DPC4 gene in various tumor types. *Cancer Res.* 1996;56:2527-2530.
24. Fujimoto A, Morita R, Hatta N, Takehara K, Takata M. p16INK4a inactivation is not frequent in uncultured sporadic primary cutaneous melanoma. *Oncogene* 1999;18:2527-2532.
25. Palacios J, Gamallo C. Mutations in the beta-catenin gene (CTNNB1) in endometrioid ovarian carcinomas. *Cancer Res.* 1998;58:1344-1347.
26. Moskaluk CA, Hruban RH, Schutte M, et al. Genomic sequencing of DPC4 in the analysis of familial pancreatic carcinoma. *Diagn Mol Pathol.* 1997;6:85-90.
27. Hussussian CJ, Struewing JP, Goldstein AM, et al. Germline p16 mutations in familial melanoma. *Nat Genet.* 1994;8:15-21.
28. Soufir N, Avril MF, Chompret A, et al. Prevalence of p16 and CDK4 germline mutations in 48 melanoma-prone families in France: The French Familial Melanoma Study Group. *Hum Mol Genet.* 1998;7:209-216.
29. Dodge JE, List AF, Futscher BW. Selective variegated methylation of the p15 CpG island in acute myeloid leukemia. *Int J Cancer.* 1998;78:561-567.
30. Norgett EE, Hatsell SJ, Carvajal-Huerta L, et al. Recessive mutation in desmoplakin disrupts desmoplakin-intermediate filament interactions and causes dilated cardiomyopathy, woolly hair and keratoderma. *Hum Mol Genet.* 2000;9:2761-2766.
31. Okabe H, Ikai I, Matsuo K, et al. Comprehensive allelotype study of hepatocellular carcinoma: potential differences in pathways to hepatocellular carcinoma between hepatitis B virus-positive and -negative tumors. *Hepatology.* 2000;31:1073-1079.
32. Soufir N, Moles JP, Vilmer C, et al. P16 UV mutations in human skin epithelial tumors. *Oncogene.* 1999;18:5477-5481.
33. Xiao W, Oefner PJ. Denaturing high-performance liquid chromatography: a review. *Hum Mutat.* 2001;17:439-474.
34. Gross E, Arnold N, Goette J, Schwarz-Boeger U, Kiechle M. A comparison of BRCA1 mutation analysis by direct sequencing, SSCP and DHPLC. *Hum Genet.* 1999;105:72-78.
35. Riggins GJ, Kinzler KW, Vogelstein B, Thiagalingam S. Frequency of Smad gene mutations in human cancers. *Cancer Res.* 1997;57:2578-2580.

36. Ohta M, Nagai H, Shimizu M, et al. Rarity of somatic and germline mutations of the cyclin-dependent kinase 4 inhibitor gene, CDK4I, in melanoma. *Cancer Res.* 1994;54:5269-5272.
37. Wang X, Egan KM, Gragoudas ES, Kelsey KT. Constitutional alterations in p16 in patients with uveal melanoma. *Melanoma Res.* 1996;6:405-410.
38. Kamb A, Gruis NA, Weaver-Feldhaus J, et al. A cell cycle regulator potentially involved in the genesis of many tumor types. *Science.* 1994;264:436-440.
39. Piccinin S, Doglioni C, Maestro R, et al. p16/CDKN2 and CDK4 gene mutations in sporadic melanoma development and progression. *Int J Cancer.* 1997;74:26-30.
40. van der Velden PA, Metzelaar-Blok JA, Bergman W, et al. Promoter hypermethylation: a common cause of reduced p16(INK4a) expression in uveal melanoma. *Cancer Res.* 2001;61:5303-5306.
41. Roth S, Laiho P, Salovaara R, Launonen V, Aaltonen LA. No SMAD4 hypermethylation in colorectal cancer. *Br J Cancer.* 2000;83:1015-1019.
42. Qian X, Jin L, Kulig E, Lloyd RV. DNA methylation regulates p27kip1 expression in rodent pituitary cell lines. *Am J Pathol.* 1998;153:1475-1482.
43. Osada H, Tatematsu Y, Masuda A, et al. Heterogeneous transforming growth factor (TGF)-beta unresponsiveness and loss of TGF-beta receptor type II expression caused by histone deacetylation in lung cancer cell lines. *Cancer Res.* 2001;61:8331-8339.
44. Kibel AS, Christopher M, Faith DA, Bova GS, Goodfellow PJ, Isaacs WB. Methylation and mutational analysis of p27(kip1) in prostate carcinoma. *Prostate.* 2001;48:248-253.
45. Cree IA. Cell cycle and melanoma: two different tumours from the same cell type. *J Pathol.* 2000;191:112-114.

Current Eye Research
2003, Vol. 27, No. 2, pp. 69–74

0271-3683/03/2702-069$16.00

Mini-review

Molecular genetics of uveal melanoma

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Abstract

In the past decade, there have been impressive advances in our understanding of chromosomal, genetic and molecular alterations that occur in uveal melanoma. Nevertheless, a coherent picture of the molecular pathogenesis of this eye cancer is yet to emerge. Herein, we review the findings to date, discuss the insights they provide, and suggest future directions for molecular research in uveal melanoma.

Introduction

Uveal melanoma is the most common malignant neoplasm of the eye, with an incidence of about seven cases per million per year in the U.S.,[1] and a five year mortality rate of up to 53%.[2] Uveal melanoma is rarely hereditary, and this lack of familial cases has hindered the search for disease-causing genes. Therefore, investigators have relied on other chromosomal, genetic and molecular approaches to study uveal melanoma. Herein, we review these findings and discuss the insights they provide into the molecular pathogenesis of uveal melanoma.

Cytogenetic analysis

Cytogenetic analysis provides an overview of structural chromosomal changes in tumor cells. Gain or loss of chromosomal material can suggest the presence of oncogenes or tumor suppressor genes, respectively. Over 50% of uveal melanomas demonstrate loss of all or part of chromosome 3, which has been strongly linked to the development of metastatic disease.[3–6] Extra copies of chromosome 8 or gain of material on the q arm of chromosome 8 occurs in 42–54% of tumors and has also been linked to metastatic death.[4,7] Alteration of chromosome 6 is also observed frequently and may correlate with better prognosis.[7,8] Less frequent cytogenetic rearrangements are observed on chromosome 9 and 21.[7,8] Despite the identification of these non-random cytogenetic changes, it remains unclear these gross chromosomal alterations play a direct role in the development of uveal melanoma by impacting the expression of cancer-related genes or whether they are simply secondary changes that occur during malignant progression.

SKY and CGH

Advances in cytogenetic techniques have allowed more precise identification of changes in chromosomal material. Spectral karyotyping (SKY) is a 24-color, multi-chromosomal painting assay that allows the visualization of all chromosomes in one experiment. SKY can detect equivocal or complex chromosomal rearrangements, and it can identify the chromosomal origins of marker chromosomes and other extra-chromosomal structures.[9] Naus and colleagues performed SKY analysis in two uveal melanoma cell lines and five primary uveal melanoma and showed that changes on chromosomes 6 and 8 occurred more frequently than previously observed with cytogenetic analysis.[10] In addition, an alteration on chromosome 17 – der(17)t(7;17)(?;q?) – was detected by SKY that was not identified by conventional cytogenetics. This chromosome 17 rearrangement could potentially involve the tumor suppressors p53 or NF1.

Comparative genomic hybridization (CGH) allows rapid screening for gains and losses of DNA copy number across

Received: March 13, 2003
Accepted: June 12, 2003

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the entire genome by comparing test and reference DNA samples that are differentially labeled.[11] CGH has validated previous cytogenetic results in uveal melanoma, including loss of chromosome 3, gain of chromosome 6p, loss of chromosome 6q, and gain of chromosome 8q.[12,13] The smallest amplified regions on 6p and 8q by CGH were 6pter->p21 and 8q24->qter, respectively.[12] CGH has identified changes that were not detected by cytogenetic analysis, including a small intrachromosomal deletion at chromosome 3q13–3q21,[10] deletions involving a tumor suppressor region at chromosome 9p,[12] and losses on chromosome 1p that were linked with metastatic disease.[5]

Loss of heterozygosity

Loss of heterozygosity (LOH) analysis is yet another step closer toward evaluating individual genes. LOH analysis utilizes polymorphic DNA markers to investigate allelic loss of specific DNA loci.[14] Loss of heterozygosity near a potential tumor suppressor gene suggests that at least one copy of the gene is deleted in a clonal manner within the tumor. Scholes and colleagues performed LOH analysis in 105 uveal melanomas using markers on chromosome 3, and they found loss of one allele for all informative markers (consistent with monosomy or loss of the entire chromosome) in 51% of cases, and regional loss of heterozygosity in another 6% of cases.[15] Monosomy 3 was associated with epithelioid cytology, intratumoral vascular loops, larger tumor diameter, ciliary body involvement, and metastatic death. Parella and colleagues screened every autosomal chromosome and the X chromosome in 50 primary uveal melanomas and found allelic loss of all informative markers on chromosome 3 in 59% of cases.[16] Allelic imbalance on chromosome 8q was observed in 60% of cases, and allelic loss on chromosome 6p was found in 28% of cases. Interestingly, this study found that changes on chromosomes 3 and 6 were usually mutually exclusive, but the 8q imbalance could occur in association with either of the other two changes, suggesting a bifurcation in the pathway of tumor progression.[16] These LOH studies confirmed earlier cytogenetic findings and indicated that some alterations, such as loss of chromosome 3, may be even more common than prior estimates. The greater sensitivity of detecting genetic loss with LOH compared to other techniques is probably due to the fact that it can detect not only monosomy but also acquired isodisomy (functional monosomy resulting from loss and duplication of the remaining chromosome 3).[17]

Loss of all or part of chromosome 3 appears to be the most common cytogenetic change in uveal melanoma, suggesting that one or more tumor suppressor genes important in uveal melanoma may reside on this chromosome. LOH studies have attempted to define the smallest region(s) of chromosomal loss as a first step towards identifying specific cancer-related genes. One study identified two distinct regions of frequent deletion located at 3q24–q26 and 3p25.[18] The second deletion spanned a region of about 2.5 megabases but did not include the von Hippel-Lindau tumor suppressor gene. The thyroid hormone receptor beta gene, which could potentially act as a tumor suppressor, underwent LOH in 60% of tumors,[19] but further studies have not confirmed a functional significance to this finding. The transforming growth factor beta (TGFβ) receptor II gene is located within a commonly deleted region at chromosome 3p22 and was recently found to undergo LOH in 6 of 19 of tumors.[20] This study observed abnormalities in the TGFβ pathway in 61% of uveal melanomas. However, mutations in the TFGβ receptor gene, which would directly implicate this as a tumor suppressor locus, have not been reported.

Cytogenetic analysis, SKY, CGH and LOH analysis have yielded insights into non-random DNA changes in uveal melanoma, but these techniques have not led to the identification of specific mutations in cancer-related genes. Therefore, some investigators have undertaken a "candidate gene" approach in which known cancer genes are surveyed for tumor-related mutations and abnormal expression patterns. We will review the major findings to date.

p53 and HDM2

The p53 tumor suppressor detects abnormalities associated with neoplastic transformation, such as excessive proliferation and DNA damage, and it can activate an intrinsic apoptotic program to eliminate transformed cells.[21] Therefore, cancer cells are under selective pressure to disrupt the p53 pathway, and p53 itself is mutated in over half of all human cancers. However, the role of p53 mutations in uveal melanoma is controversial. Some early studies found increased immunohistochemical expression of p53 (indicating possible mutation) in some uveal melanomas, but most of these tumors had been irradiated.[22] We recently showed that irradiation strongly increases p53 expression in uveal melanomas and that this does not necessarily indicate the presence of mutant p53.[23] A few tumors have been shown to harbor potential mutations within the p53 gene,[24] but most larger studies of untreated tumors suggest that p53 mutations are uncommon in uveal melanomas.[25]

HDM2, the human homologue of murine double minute 2 (MDM2), is an inhibitor of p53 that targets it for degradation.[26] HDM2 is overexpressed in some p53(+) tumors, suggesting that high levels of HDM2 can functionally block the p53 pathway.[27] Several groups have investigated HDM2 expression in uveal melanoma. We found that over 95% of untreated tumors demonstrated strong immunohistochemical expression of HDM2.[25] Further, Coupland and co-workers showed that increased HDM2 expression was associated with poor outcome.[28] As functional evidence that HDM2 is important for survival of uveal melanoma cells, we found that blocking HDM2 with a small molecule inhibitor caused rapid onset of apoptosis.[29] Thus, the p53 pathway may be at least partially inhibited in uveal melanomas by overexpression of HDM2, but the mechanism for this increased expression remains unclear.

Rb-p16Ink4a-Cyclin D pathway

The retinoblastoma protein (Rb) pathway suppresses tumorigenesis by regulating cell cycle progression, senescence and differentiation.[30] Virtually all cancers are thought to harbor mutations in the Rb pathway.[31] Mutation of the Rb gene is found in only a small subset of cancers, such as retinoblastoma. About 20% of uveal melanomas were found to have LOH near the Rb locus at chromosome 13q.14,[32] but there is little evidence that Rb is mutated in uveal melanoma. The Rb protein has been expressed in all untreated uveal melanomas that we examined so far.[25] Further, we are not aware of any reports of documented Rb gene mutations in a uveal melanoma.

Mutations elsewhere in the Rb pathway can functionally inactivate Rb by keeping it hyperphosphorylated. The fact that Rb is hyperphosphorylated in most primary uveal melanomas suggests that this is the primary mechanism for inhibiting the Rb pathway in this cancer.[33] Cyclin D1, which activates the Rb kinase CDK4, is strongly expressed in over 40% of uveal melanomas and has been shown to block the active repressor function of Rb.[33] Increased cyclin D1 expression has been associated with larger tumor size, epithelioid cell type, and poor prognosis.[25,28] p16Ink4a is an inhibitor of cyclin D-mediated phosphorylation.[31] Although germline mutations in the p16Ink4a gene are exceedingly rare in uveal melanoma patients,[34–37] several studies suggest that somatic silencing of the p16Ink4a gene may occur in some tumors. Merbs and co-workers found LOH at the p16Ink4a locus in 24% of tumors, half of which had homozygous deletion.[38] The promoter was methylated in another 6% of cases. The authors concluded that p16Ink4a was inactivated in up to 27% of the tumors. However, gene expression was not directly assessed, and since no intragenic mutations were observed, it is possible that the deletions targeted other nearby tumor suppressor genes such as p14ARF and p15Ink4b. In another study, methylation of the p16Ink4a promoter was observed in 32% of primary uveal melanomas,[39] further suggesting that silencing of p16Ink4a may occur in up to a third of uveal melanomas.

C-myc

C-myc regulates cellular proliferation, apoptosis, and cell differentiation, and it has been implicated as an oncogene in selected types of human cancer.[40] The c-myc gene is located at chromosome 8q24, within the region of frequent overrepresentation in uveal melanoma. By immunohistochemistry, about 70% of uveal melanomas strongly express c-myc,[41,42] and there may be an association between increased c-myc expression and improved survival.[41] As further evidence that c-myc is specifically altered in uveal melanoma, one study found that the c-myc gene was amplified or overrepresented in 90% of primary tumors, and c-myc amplification was associated with larger tumor size.[43] Nevertheless, it remains possible that these amplification events actually target a nearby gene or that they represent a survival response due to cellular stress in transformed cells.

Bcl-2

Bcl-2 is an anti-apoptotic factor and the namesake of a family of pro- and anti-apoptotic proteins that interact in a complex manner to regulate apoptosis by the intrinsic mitochondrial pathway.[44] Bcl-2 is overexpressed in many cancer types as a mechanism for inhibiting apoptosis. Overexpression of Bcl-2 by immunohistochemical analysis has been reported in the vast majority of uveal melanomas.[25,41,45] As evidence that this strong Bcl-2 expression may be required for survival of uveal melanoma cells, we showed that inhibition of Bcl-2 by a selective small molecule inhibitor led to rapid induction of apoptosis.[29] Interestingly, elevated Bcl-2 expression may be an intrinsic property of cells of melanocytic lineage, potentially explaining the profound resistance to therapy-induced apoptosis that is observed in all forms of melanoma.[46]

Telomerase

Telomerase is a ribonucleoprotein polymerase capable of extending telomeres that are normally lost during successive cell divisions. Telomerase is not normally active in somatic cells, but overexpression of telomerase occurs in some cancers and can stabilize telomeres and prevent the induction of cellular senescence. Telomerase activity was detected in 14 of 14 uveal melanomas by *in situ* hybridization.[47] Since a putative repressor of telomerase is located on chromosome 3, it would be interesting to determine whether the frequent genetic loss on chromosome 3 may provide the tumor a selective advantage by promoting telomerase activity.

BRCA2

BRCA2 is a DNA damage repair protein, and mutation of the BRCA2 gene can lead to accumulation of DNA damage and a propensity for neoplastic transformation. Germline BRCA2 mutations have been found in up to 3% of uveal melanoma patients.[48,49] While this finding strongly implicates a role for DNA damage repair defects in the pathogenesis of uveal melanoma in these patients, these studies were limited to selected patients that were highly likely to have a genetic predisposition melanoma, so the role of BRCA2 in the vast majority of sporadic uveal melanoma patients remains unclear.

NF1

The NF1 tumor suppressor locus on chromome 17 was deleted and NF1 protein expression was lacking in one of 38 tumors using dual color fluorescence in situ hybridization.[50] These findings suggest that NF1 may infrequently be the target of mutations in uveal melanoma and may explain the

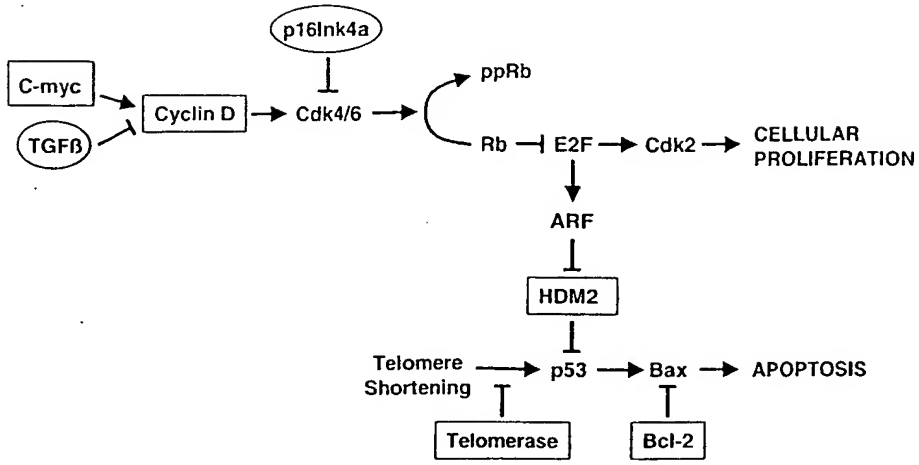

Figure 1. Many of the molecular abnormalities that have been identified in uveal melanoma are likely to inhibit the Rb-p53 tumor suppressor pathway. The retinoblastoma protein (Rb) blocks cell proliferation primarily by inhibiting the transcription factor E2F, which activates factors involved in cell division such as CDK2. Rb is itself inhibited by cyclin D-mediated phosphorylation. Overexpression of the oncogenes c-myc and cyclin D, inactivation of the tumor suppressor p16Ink4a, or inhibition of the TGFβ tumor suppressor pathway can promote cyclin D-mediated phosphorylation, causing Rb to be maintained in a hyperphosphorylated, inactive state. Inactivation of Rb normally triggers an apoptotic response in part by activation of the alternate reading frame protein (ARF), which activates p53 by blocking the p53 inhibitor HDM2. Overexpression of HDM2 can short circuit this tumor suppressor pathway and prevent p53 from becoming active. Overexpression of Bcl-2 and other anti-apoptotic proteins downstream of p53 can block pro-apoptotic proteins that are activated by p53, such as Bax, further inhibiting the apoptotic drive. Shortening of telomeres normally functions as a cellular "clock" to limit the number of cell divisions that a cell can undergo before entering senescence by activating the p53 pathway. Immortalized tumor cells often overexpress telomerase to prevent the shortening of telomeres. The factors that are illustrated in boxes are frequently overexpressed in uveal melanoma, and the factors in ovals are frequently inactivated. See text for details.

clinical association between neurofibromatosis type 1 and uveal melanoma.

Conclusions

A variety of cytogenetic, genetic and molecular findings have provided insights into the molecular pathogenesis of uveal melanoma. However, a coherent picture has not yet emerged (Fig. 1). Cytogenetic changes such as loss of chromosome 3 occur frequently but have not been shown to target specific genes. Hence, it is unclear whether these gross chromosomal abnormalities play a causal role or are simply markers for disease progression. LOH analysis has identified specific regions of DNA loss. But here again, loss of heterozygosity alone has not implicated the involvement of specific tumor suppressor genes. The Rb pathway appears to be functionally inhibited in the vast majority of uveal melanomas by hyperphosphorylation of the Rb protein. Overexpression of cyclin D1 and inactivation of p16Ink4a appear to be the most common mechanisms for maintaining Rb in a phosphorylated state. The p53 pathway is functionally blocked in most uveal melanomas, and this inhibition may be due at least in part to overexpression of the anti-apoptotic proteins HDM2 and Bcl-2. C-myc is frequently amplified and overexpressed in uveal melanoma, although we still await functional validation that this is a primary rather than secondary change. Future studies should focus on the development of genetically targeted animal models and functional studies in primary uveal melanoma cells in order to determine which of the myriad changes is important in the initiation and progression of uveal melanoma.

Acknowledgements

Research funding was provided by the National Eye Institute (K08 EY00382 and R01 EY13169) and Research to Prevent Blindness, Inc.

References

1. Egan KM, Seddon JM, Glynn RJ, Gragoudas ES, Albert DM. Epidemiologic aspects of uveal melanoma. *Surv Ophthalmol.* 1988;32:239–251.
2. Diener-West M, Hawkins BS, Markowitz JA, Schachat AP. A review of mortality from choroidal melanoma. II. A meta-analysis of 5-year mortality rates following enucleation, 1966 through 1988. *Arch Ophthalmol.* 1992;110: 245–250.
3. Horsthemke B, Prescher G, Bornfeld N, Becher R. Loss of chromosome 3 alleles and multiplication of chromosome 8 alleles in uveal melanoma. *Genes Chromosomes Cancer.* 1992;4:217–221.
4. Sisley K, Rennie IG, Parsons MA, Jacques R, Hammond DW, Bell SM, Potter AM, Rees RC. Abnormalities of chro-

mosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. *Genes Chromosomes Cancer*. 1997;19:22–28.

5. Aalto Y, Eriksson L, Seregard S, Larsson O, Knuutila S. Concomitant loss of chromosome 3 and whole arm losses and gains of chromosome 1, 6, or 8 in metastasizing primary uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001;42:313–317.
6. Prescher G, Bornfeld N, Hirche H, Horsthemke B, Jockel KH, Becher R. Prognostic implications of monosomy 3 in uveal melanoma. *Lancet*. 1996;347:1222–1225.
7. Prescher G, Bornfeld N, Friedrichs W, Seeber S, Becher R. Cytogenetics of twelve cases of uveal melanoma and patterns of nonrandom anomalies and isochromosome formation. *Cancer Genet Cytogenet*. 1995;80:40–46.
8. Singh AD, Boghosian-Sell L, Wary KK, Shields CL, De Potter P, Donoso LA, Shields JA, Cannizzaro LA. Cytogenetic findings in primary uveal melanoma. *Cancer Genet Cytogenet*. 1994;72:109–115.
9. Bayani JM, Squire JA. Applications of SKY in cancer cytogenetics. *Cancer Invest*. 2002;20:373–386.
10. Naus NC, van Drunen E, de Klein A, Luyten GP, Paridaens DA, Alers JC, Ksander BR, Beverloo HB, Slater RM. Characterization of complex chromosomal abnormalities in uveal melanoma by fluorescence *in situ* hybridization, spectral karyotyping, and comparative genomic hybridization. *Genes Chromosomes Cancer*. 2001;30:267–273.
11. Oga A, Kawauchi S, Izumi H, Ping LX, Furuya T, Sasaki K. New perspectives for tumor pathology provided by comparative genomic hybridization. *Int J Clin Oncol*. 2002;7:133–137.
12. Speicher MR, Prescher G, du Manoir S, Jauch A, Horsthemke B, Bornfeld N, Becher R, Cremer T. Chromosomal gains and losses in uveal melanomas detected by comparative genomic hybridization. *Cancer Res*. 1994; 54:3817–3823.
13. Gordon KB, Thompson CT, Char DH, O'Brien JM, Kroll S, Ghazvini S, Gray JW. Comparative genomic hybridization in the detection of DNA copy number abnormalities in uveal melanoma. *Cancer Res*. 1994;54:4764–4768.
14. Cavenee WK, Dryja TP, Phillips RA, Benedict WF, Godbout R, Gallie BL, Murphree AL, Strong LC, White RL. Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. *Nature*. 1983;305:779–784.
15. Scholes AG, Damato BE, Nunn J, Hiscott P, Grierson I, Field JK. Monosomy 3 in uveal melanoma: Correlation with clinical and histologic predictors of survival. *Invest Ophthalmol Vis Sci*. 2003;44:1008–1011.
16. Parrella P, Sidransky D, Merbs SL. Allelotype of posterior uveal melanoma: Implications for a bifurcated tumor progression pathway. *Cancer Res*. 1999;59:3032–3037.
17. White VA, McNeil BK, Horsman DE. Acquired homozygosity (isodisomy) of chromosome 3 in uveal melanoma. *Cancer Genet Cytogenet*. 1998;102:40–45.
18. Tschentscher F, Prescher G, Horsman DE, White VA, Rieder H, Anastassiou G, Schilling H, Bornfeld N, Bartz-Schmidt KU, Horsthemke B, Lohmann DR, Zeschnigk M. Partial deletions of the long and short arm of chromosome 3 point to two tumor suppressor genes in uveal melanoma. *Cancer Res*. 2001;61:3439–3442.
19. Sisley K, Curtis D, Rennie IG, Rees RC. Loss of heterozygosity of the thyroid hormone receptor B in posterior uveal melanoma. *Melanoma Res*. 1993;3:457–461.
20. Myatt N, Aristodemou P, Neale MH, Foss AJE, Hungerford JL, Bhattacharya S, Cree IA. Abnormalities of the transforming growth factor-beta pathway in ocular melanoma. *J Pathol*. 2000;192:511–518.
21. Prives C, Hall PA. The p53 pathway. *J Pathol*. 1999; 187:112–126.
22. Janssen K, Kuntze J, Busse H, Schmid KW. p53 oncoprotein overexpression in choroidal melanoma. *Mod Pathol*. 1996;9:267–272.
23. Brantley MA, Jr., Worley L, Harbour JW. Altered expression of Rb and p53 in uveal melanomas following plaque radiotherapy. *Am J Ophthalmol*. 2002;133:242–248.
24. Tobal K, Warren W, Cooper CS, McCartney A, Hungerford J, Lightman S. Increased expression and mutation of p53 in choroidal melanoma. *Br J Cancer*. 1992;66:900–904.
25. Brantley MA, Jr., Harbour JW. Deregulation of the Rb and p53 pathways in uveal melanoma. *Am J Pathol*. 2000; 157:1795–1801.
26. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature*. 1997;387:296–299.
27. Landers JE, Cassel SL, George DL. Translational enhancement of mdm2 oncogene expression in human tumor cells containing a stabilized wild-type p53 protein. *Cancer Res*. 1997;57:3562–3568.
28. Coupland SE, Anastassiou G, Stang A, Schilling H, Anagnostopoulos I, Bornfeld N, Stein H. The prognostic value of cyclin D1, p53, and MDM2 protein expression in uveal melanoma. *J Pathol*. 2000;191:120–126.
29. Harbour JW, Worley L, Ma D, Cohen M. Transducible peptide therapy for uveal melanoma and retinoblastoma. *Arch Ophthalmol*. 2002;120:1341–1346.
30. Harbour JW, Dean DC. The Rb/E2F pathway: Emerging paradigms and expanding roles. *Genes Dev*. 2000;14:2545–2562.
31. Sherr CJ. Cancer cell cycles. *Science*. 1996;274:1672–1677.
32. Scholes AG, Liloglou T, Maloney P, Hagan S, Nunn J, Hiscott P, Damato BE, Grierson I, Field JK. Loss of heterozygosity on chromosomes 3, 9, 13, and 17, including the retinoblastoma locus, in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001;42:2472–2477.
33. Brantley MA, Jr., Harbour JW. Inactivation of retinoblastoma protein in uveal melanoma by phosphorylation of sites in the COOH-terminal region. *Cancer Res*. 2000; 60:4320–4323.
34. Soufir N, Bressac-de Paillerets B, Desjardins L, Levy C, Bombled J, Gorin I, Schlienger P, Stoppa-Lyonnet D. Individuals with presumably hereditary uveal melanoma do not harbour germline mutations in the coding regions of either

the P16INK4A, P14ARF or cdk4 genes. *Br J Cancer*. 2000;82:818–822.
35. Singh AD, Croce CM, Wary KK, Shields JA, Donoso LA, Shields CL, Huebner K, Ohta M. Familial uveal melanoma: Absence of germline mutations involving the cyclin-dependent kinase-4 inhibitor gene (p16). *Ophthalmic Genet*. 1996;17:39–40.
36. Hearle N, Damato BE, Humphreys J, Wixey J, Green H, Stone J, Easton DF, Houlston RS. Contribution of germline mutations in BRCA2, P16(INK4A), P14(ARF) and P15 to uveal melanoma. *Invest Ophthalmol Vis Sci*. 2003;44:458–462.
37. Wang X, Egan KM, Gragoudas ES, Kelsey KT. Constitutional alterations in p16 in patients with uveal melanoma. *Melanoma Res*. 1996;6:405–410.
38. Merbs SL, Sidransky D. Analysis of p16 (CDKN2/MTS-1/INK4A) alterations in primary sporadic uveal melanoma. *Invest Ophthalmol Vis Sci*. 1999;40:779–783.
39. van der Velden PA, Metzelaar-Blok JA, Bergman W, Monique H, Hurks H, Frants RR, Gruis NA, Jager MJ. Promoter hypermethylation: A common cause of reduced p16(INK4a) expression in uveal melanoma. *Cancer Res*. 2001;61:5303–5306.
40. Henriksson M, Luscher B. Proteins of the Myc network: Essential regulators of cell growth and differentiation. *Adv Cancer Res*. 1996;68:109–182.
41. Chana JS, Wilson GD, Cree IA, Alexander RA, Myatt N, Neale M, Foss AJ, Hungerford JL. c-myc, p53, and Bcl-2 expression and clinical outcome in uveal melanoma. *Br J Ophthalmol*. 1999;83:110–114.
42. Royds JA, Sharrard RM, Parsons MA, Lawry J, Rees R, Cottam D, Wagner B, Rennie IG. C-myc oncogene expression in ocular melanomas. *Graefes Arch Clin Exp Ophthalmol*. 1992;230:366–371.
43. Parrella P, Caballero OL, Sidransky D, Merbs SL. Detection of c-myc amplification in uveal melanoma by fluorescent *in situ* hybridization. *Invest Ophthalmol Vis Sci*. 2001;42:1679–1684.
44. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev*. 1999;13:1899–1911.
45. Jay V, Yi Q, Hunter WS, Zielenska M. Expression of bcl-2 in uveal malignant melanoma. *Arch Pathol Lab Med*. 1996;120:497–498.
46. McGill GG, Horstmann M, Widlund HR, Du J, Motyckova G, Nishimura EK, Lin YL, Ramaswamy S, Avery W, Ding HF, Jordan SA, Jackson IJ, Korsmeyer SJ, Golub TR, Fisher DE. Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. *Cell*. 2002;109:707–718.
47. Heine B, Coupland SE, Kneiff S, Demel G, Bornfeld N, Hummel M, Stein H. Telomerase expression in uveal melanoma. *Br J Ophthalmol*. 2000;84:217–223.
48. Scott RJ, Vajdic CM, Armstrong BK, Ainsworth CJ, Meldrum CJ, Aitken JF, Kricker A. BRCA2 mutations in a population-based series of patients with ocular melanoma. *Int J Cancer*. 2002;102:188–191.
49. Iscovich J, Abdulrazik M, Cour C, Fischbein A, Pe'er J, Goldgar DE. Prevalence of the BRCA2 6174 del T mutation in Israeli uveal melanoma patients. *Int J Cancer*. 2002;98:42–44.
50. Foster WJ, Fuller CE, Perry A, Harbour JW. Status of the NF1 tumor suppressor locus in uveal melanoma. *Arch Ophthalmol*. 2003;in press.

Current Eye Research
2003, Vol. 27, No. 2, pp. 75–83

0271-3683/03/2702-075$16.00

Expression of glycosaminoglycans during development of the rat retina

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Abstract

Purpose. To investigate the spatiotemporal expression of glycosaminoglycans during development of the rat retina.

Methods. Hyaluronan and sulfated glycosaminoglycans, including chondroitin sulfate, heparan sulfate and keratan sulfate were detected using biotinylated hyaluronan binding protein, immunohistochemical analysis, respectively, in the rat retina at various stages of development.

Results. Hyaluronan was expressed in the nerve fiber layer, inner plexiform layer and outer plexiform layer during early postnatal stages (postnatal day 1–14; P1–P14) and was undetectable after P21. In contrast, hyaluronan was faintly observed in the photoreceptor layer on P7, and gradually increased up to P49. The spatiotemporal expression pattern of chondroitin sulfate was similar to that of hyaluronan. Heparan sulfate was also detected in the nerve fiber layer, inner plexiform layer and outer plexiform layer during early postnatal stages (P1–P14). In addition, heparan sulfate was expressed in the inner limiting membrane during all stages of development. Keratan sulfate was not detected in the retina at any stage of development.

Conclusions. Hyaluronan, chondroitin sulfate and heparan sulfate are expressed in nerve fiber-rich layers during early postnatal stages and may regulate neurite outgrowth. In adulthood, both hyaluronan and chondroitin sulfate are expressed in the photoreceptor layer and may consist of the interphotoreceptor matrix. In addition, heparan sulfate is expressed in the inner limiting membrane throughout the various stages of development and may be associated with the structure of the inner limiting membrane.

Keywords: proteoglycan; glycosaminoglycan; hyaluronan; retina; development

Introduction

Extracellular matrix molecules play important roles in the control of cell proliferation, migration, differentiation, and maintenance of morphogenic structures.[1–3] Proteoglycans are one group of extracellular matrix molecules composed of a core protein molecule to which glycosaminoglycans are covalently linked as side chains. Glycosaminoglycans are large polymers that consist of repeating disaccharide units, and classified into 4 main forms including chondroitin sulfate, heparan sulfate, keratan sulfate and hyaluronan. Chondroitin sulfate and heparan sulfate proteoglycans were found to be expressed during development of the retina.[4] Although many studies have focused on the core proteins of proteoglycans, glycosaminoglycans have been implicated in many biological functions, such as binding to extracellular matrix proteins and growth factors, and appeared to play important regulatory roles in many processes, such as cell adhesion, cell migration and differentiation.[5] We now report expression of each glycosaminoglycan which changes markedly and specifically during development of the rat retina. The physiological significance of the results is discussed.

Received: December 24, 2002
Accepted: March 6, 2003

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Lack of BRAF Mutation in Primary Uveal Melanoma

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PURPOSE. BRAF T1796A activating mutations have been found in a high proportion of cutaneous melanomas, cutaneous nevi, and papillary thyroid carcinoma and in a small fraction of other cancers. This study was designed to investigate the incidence of BRAF T1796A mutation in uveal melanoma.

METHODS. Twenty-nine formalin-fixed, paraffin-embedded posterior uveal melanomas were included in the study. DNA was extracted from the paraffin sections followed by PCR amplification of exon 15 and detection of the common BRAF missense mutation (T→A transversion at nucleotide 1796) using restriction enzyme analysis.

RESULTS. Although positive cutaneous melanoma control cell lines harbored the T1796A BRAF mutation, none of the 29 uveal melanomas harbored the mutation.

CONCLUSIONS. These data suggest that BRAF T1796A activating mutation is not common in primary uveal melanoma. These findings are in accord with known differences in tumorigenesis between uveal and cutaneous melanomas. (*Invest Ophthalmol Vis Sci.* 2003;44:2876–2878) DOI:10.1167/iovs.02-1329

Uveal melanoma is the most common form of primary eye cancer in adults, with an annual incidence of six to seven cases per million.[1] It accounts for 80% of the noncutaneous melanomas and for 13% of all deaths caused by melanoma.[2] This tumor carries up to a 50% 5-year mortality from metastasis that is associated with both histologic and demographic prognostic factors such as cell type, tumor diameter and location, chromosomal aberration, age, and sex.[1,3,4]

Uveal and cutaneous melanomas originate from a common precursor cell, the melanocyte, which migrates from the neural crest to the respective site during the embryonic development period.[5] The similar genetic background and some common histologic characteristics suggest a similar pathogenesis in these tumors.[6,7] Although risk factors such as fair skin complexion and blue iris color may be shared,[4] UV radiation and sunlight effect appears to play a significant role only in cutaneous melanoma.[8] Furthermore, uveal melanoma metastasizes hematogenously, with the liver frequently affected,[9] whereas cutaneous melanoma tends to spread through the lymphatic system, usually affecting the regional lymph nodes.[10]

Unlike cutaneous melanoma, little is known about the underlying molecular pathogenesis of uveal melanoma. Loss of gene function due to deletions or inactivating mutations, as well as gain-of-function mutations is the hallmark of cancer cells.[11] To date, no oncogenes or tumor-suppressor genes have been linked to uveal melanoma. Cytogenetic analyses of uveal melanoma have identified chromosome 3 monosomy and increased chromosome 8, short arm, copy number in more than 50% of the tumors.[12,13] This alteration also correlates significantly with metastasis and decreased survival.[1,14]

The importance of oncogenic mutations in the RAS/ RAF/ MEK/ERK pathway has been well documented in human cancer. More than 15% of all human cancers harbor point mutations of RAS.[15] Constant activation of this pathway provides a potent promitogenic force, resulting in abnormal proliferation and differentiation in many human cancers.[16] The association between RAS mutations and human uveal melanomas was investigated in several studies.[17,18] Soparker et al.[18] screened Ha-ras, Ki-ras, and N-ras at codons 12, 13, and 61 and could not find any mutations. It is still not known whether other genes in the RAS/ RAF/ MEK/ERK pathway have a role in the development of uveal melanoma.

Mutations in one of the RAF genes, BRAF, have been recently discovered in the majority of cutaneous melanomas,[15] cutaneous nevi,[19] and papillary thyroid carcinoma[20] and to a lesser extent in other cancers.[15,21] The predominant mutation reported in cutaneous melanoma and cutaneous nevi was a thymine-to-adenine (T→A) transversion at nucleotide position 1796 (corresponding to an amino-acid swap of glutamate for valine at residue 599; V599E). This transversion resulted in constant activation of BRAF and, in turn, of the MEK/ERK pathway. To screen for a possible shared etiologic factor between uveal and cutaneous melanomas, we screened 29 cases of primary posterior uveal melanoma tumors for the T1796A BRAF mutation.

MATERIALS AND METHODS

Pathologic Specimens

Formalin-fixed, paraffin-embedded sections from 29 posterior uveal melanomas were included in the study. The sections were collected from the Wilmer Eye Institute at the Johns Hopkins University School of Medicine (Baltimore, MD) and the ophthalmic pathology laboratory of the Hadassah University Hospital (Jerusalem, Israel). All tumor samples were removed as part of the patient's treatment and with local ethics committee approval for use of the tissue in this study. The study protocol adhered to the tenets of the Declaration of Helsinki.

DNA Extraction

Tumor tissue was microdissected from an area in the sections with more than 75% malignant cells. DNA was purified by standard phenol-chloroform extraction followed by ethanol precipitation.[13]

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Submitted for publication December 28, 2002; revised February 12, 2003; accepted February 21, 2003.

Disclosure: **Y. Cohen**, None; **N. Goldenberg-Cohen**, None; **P. Parrella**, None; **I. Chowers**, None; **S.L. Merbs**, None; **J. Pe'er**, None; **D. Sidransky**, None

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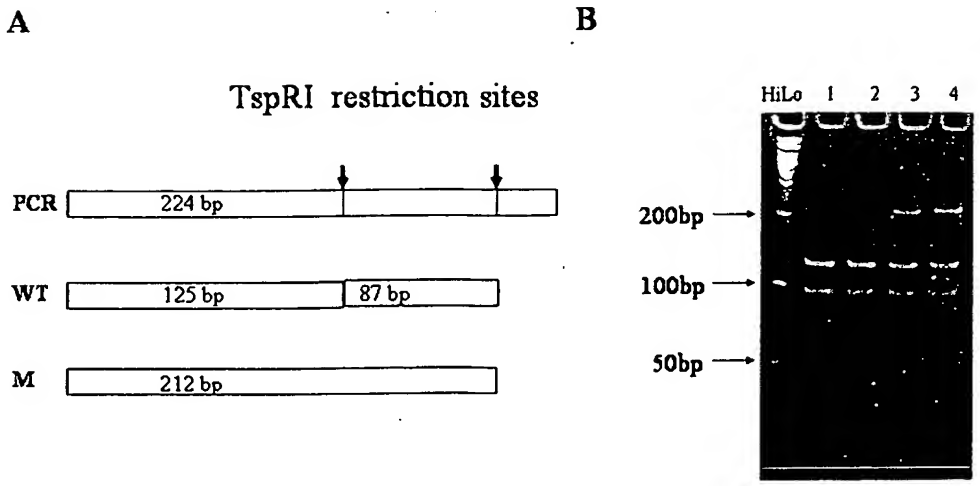

FIGURE 1. Restriction enzyme analysis of the T1796A mutation in exon 15 of the BRAF gene. (A) The presence of the T1796A mutation was determined using the restriction enzyme *Tsp*RI. Exon 15 was amplified by PCR and digested with *Tsp*RI. The T→A base transversion eliminates a *Tsp*RI restriction site within the 224-bp amplified product. PCR, uncut PCR product; WT, wild-type allele (T1796); M, mutant allele (A1796). (B) Digestion products were electrophoresed on 10% polyacrylamide gel and visualized with ethidium bromide stain. Hilo: molecular weight marker; *lanes 1* and *2*: negative cases; *lanes 3* and *4*: positive control cell lines.

BRAF Exon 15 PCR Amplification

Approximately 100 ng of total cellular DNA was used in each PCR amplification. The PCR was performed using specific BRAF exon 15 primers: forward primer: TCATAATGCTTGCTCTGATAGGA; reverse primer: GGCCAAAAATTTAATCAGTGGA, as described elsewhere.[15] A step-down PCR protocol was used as follows: 95°C for 2 minutes, 1 cycle; 95°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, 2 cycles; 95°C for 1 second, 58°C for 1 minute, 72°C for 1 minute, 2 cycles; 95°C, for 1 minute, 56°C for 1 minute, and 72°C for 1 minute for 30 cycles and a final extension at 72°C for 5 minutes.

Analysis of BRAF T1796A Mutations

Analysis of BRAF T1796A mutations was performed as described previously.[20] Briefly, 10 μL of PCR product was incubated with 1 μL of *Tsp*RI restriction enzyme (10 U/ μL New England Biolabs, Beverly, MA) in a 30-μL reaction volume overnight at 65°C. The same reaction replacing the enzyme with deionized (d)H_2O was used as negative control. The samples were loaded and run on a nondenaturing 10% polyacrylamide gel. The gels were stained with ethidium bromide, and the bands were visualized under a UV lamp.

RESULTS

To detect BRAF mutations in posterior uveal melanoma, we screened the gene for the T1796A transversion by PCR and restriction enzyme analysis. *Tsp*RI digestion of the PCR fragment yielded three major bands at 125, 87, and 12 bp in the wild-type allele. The T1796A mutation abolished one restriction site, resulting in a prominent 212-bp band from the mutant allele and residual bands from the normal allele (Fig. 1). As positive controls for the BRAF T1796A mutation, we tested the cutaneous melanoma cell lines HTB71, HTB72, and A2058 with known mutation; ME180 (cervical cancer) and HCT116 (colon cancer) served as the negative control. All 29 cases of uveal melanoma screened were negative for the BRAF T1796A mutation. This hot spot was chosen because the reported BRAF-activating mutations in cutaneous nevi and cutaneous melanoma occur almost exclusively in this position.[15,19]

DISCUSSION

Although advances in molecular genetics have made possible the identification of genetic changes and particular mutant genes in human tumors, relatively little is known about the molecular genetic alterations leading to the development of uveal melanoma. In an attempt to better understand the molecular events that lead to uveal melanoma, we searched for a BRAF mutation that has been found to occur in up to 80% of cutaneous nevi and cutaneous melanomas.[15,19]

Although common in cutaneous nevi and cutaneous melanoma, the T1796A BRAF mutation was absent in uveal melanomas. Because we did not sequence the complete exon 15 in the present study, we cannot exclude completely the presence of other less common BRAF mutations (V599D, V599K, V599R)[15,19] in uveal melanoma. However, the very common T1796A (V599E) BRAF mutation in cutaneous nevi and cutaneous melanomas does not play a role in the pathogenesis of uveal melanoma. This result is in accord with the known epidemiologic and histologic differences previously described between these two melanoma subtypes.[3,9,22] It is conceivable that uveal melanoma arises from a series of genetic changes divergent from those of cutaneous melanoma. Although chromosome 3 monosomy and 8q trisomy are common in uveal melanoma, they are rarely observed in cutaneous melanoma.[2] Integrin expression, which is essential for growth and metastatic capacity of cutaneous melanoma cells, as well as the expression of melanoma-associated antigens and the melanocortin-1 receptor, differ markedly between uveal and cutaneous melanomas.[3,23-25] Our findings thus support further genetic diversity between cutaneous and uveal melanomas.

References

1. Prescher G, Bornfeld N, Hirche H, Horsthemke B, Jockel KH, Becher R. Prognostic implications of monosomy 3 in uveal melanoma. *Lancet.* 1996;347:1222-1225.
2. Edmunds SC, Kelsell DP, Hungerford JL, Cree IA. Mutational analysis of selected genes in the TGFbeta, Wnt, pRb, and p53 pathways in primary uveal melanoma. *Invest Ophthalmol Vis Sci.* 2002;43:2845-2851.
3. Iwamoto S, Burrows RC, Kalina RE, et al. Immunophenotypic differences between uveal and cutaneous melanomas. *Arch Ophthalmol.* 2002;120:466-470.
4. Gallagher RP, Elwood JM, Rootman J, et al. Risk factors for ocular melanoma: Western Canada Melanoma Study. *J Natl Cancer Inst.* 1985;74:775-778.
5. Soufir N, Bressac-de Paillerets B, Desjardins L, et al. Individuals with presumably hereditary uveal melanoma do not harbour germline mutations in the coding regions of either the P16INK4A, P14ARF or cdk4 genes. *Br J Cancer.* 2000;82:818-822.
6. Tucker MA, Shields JA, Hartge P, Augsburger J, Hoover RN, Fraumeni JF Jr. Sunlight exposure as risk factor for intraocular malignant melanoma. *N Engl J Med.* 1985;313:789-792.
7. Rootman J, Gallagher RP. Color as a risk factor in iris melanoma. *Am J Ophthalmol.* 1984;98:558-561.

8. Guenel P, Laforest L, Cyr D, et al. Occupational risk factors, ultraviolet radiation, and ocular melanoma: a case-control study in France. *Cancer Causes Control.* 2001;12:451–459.
9. Didolkar MS, Elias EG, Barber NA, Moore RH. Biologic behavior of ocular malignant melanoma and comparison with melanoma of the head and neck. *Am J Surg.* 1980;140:522–526.
10. Chang AE, Karnell LH, Menck HR. The National Cancer Data Base report on cutaneous and noncutaneous melanoma: a summary of 84,836 cases from the past decade. The American College of Surgeons Commission on Cancer and the American Cancer Society. *Cancer.* 1998;83:1664–1678.
11. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell.* 2000; 100:57–70.
12. Horsthemke B, Prescher G, Bornfeld N, Becher R. Loss of chromosome 3 alleles and multiplication of chromosome 8 alleles in uveal melanoma. *Genes Chromosomes Cancer.* 1992;4:217–221.
13. Parrella P, Sidransky D, Merbs SL. Allelotype of posterior uveal melanoma: implications for a bifurcated tumor progression pathway. *Cancer Res.* 1999;59:3032–3037.
14. Scholes AG, Liloglou T, Maloney P, et al. Loss of heterozygosity on chromosomes 3, 9, 13, and 17, including the retinoblastoma locus, in uveal melanoma. *Invest Ophthalmol Vis Sci.* 2001;42:2472–2477.
15. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature.* 2002;417:949–954.
16. Avruch J, Khokhlatchev A, Kyriakis JM, et al. Ras activation of the Raf kinase: tyrosine kinase recruitment of the MAP kinase cascade. *Recent Prog Horm Res.* 2001;56:127–155.
17. Mooy CM, Van der Helm MJ, Van der Kwast TH, De Jong PT, Ruiter DJ, Zwarthoff EC. No N-ras mutations in human uveal melanoma: the role of ultraviolet light revisited. *Br J Cancer.* 1991;64:411–413.
18. Soparker CN, O'Brien JM, Albert DM. Investigation of the role of the ras protooncogene point mutation in human uveal melanomas. *Invest Ophthalmol Vis Sci.* 1993;34:2203–2209.
19. Pollock PM, Harper UL, Hansen KS, et al. High frequency of BRAF mutations in nevi. *Nat Genet.* 2003;33:19–20.
20. Cohen Y, Xing M, Mambo E, et al. BRAF mutation in papillary thyroid carcinoma. *J Natl Cancer Inst.* 2003;95:625–627.
21. Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. *Nature.* 2002;418:934.
22. Shors AR, Iwamoto S, Doody DR, Weiss NS. Relationship of uveal and cutaneous malignant melanoma in persons with multiple primary tumors. *Int J Cancer.* 2002;102:266–268.
23. Mooy CM, De Jong PT. Prognostic parameters in uveal melanoma: a review. *Surv Ophthalmol.* 1996;41:215–228.
24. Marshall JF, Rutherford DC, Happerfield L, et al. Comparative analysis of integrins in vitro and in vivo in uveal and cutaneous melanomas. *Br J Cancer.* 1998;77:522–529.
25. Metzelaar-Blok JA, ter Huurne JA, Hurks HM, Keunen JE, Jager MJ, Gruis NA. Characterization of melanocortin-1 receptor gene variants in uveal melanoma patients. *Invest Ophthalmol Vis Sci.* 2001; 42:1951–1954.

Integrin Expression in Uveal Melanoma Differs From Cutaneous Melanoma

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Purpose. During the process of metastasis, changes in cell–cell and cell–matrix contacts occur; therefore, expression of integrins, a superfamily of adhesion molecules, may be important. Expression of integrins has been studied extensively in cutaneous melanoma. Because it is known that uveal melanoma has a metastatic behavior different from cutaneous melanoma, the authors investigated integrin expression in uveal melanoma.

Methods. The authors used monoclonal antibodies recognizing integrin subunits α1-6, αv, β1, and β4 and integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ on frozen sections of 32 human primary uveal melanomas and 4 metastases, followed by an avidin-biotin-peroxidase complex–immunoperoxidase technique.

Results. As in cutaneous melanoma, α4 expression was rare, but most lesions expressed α3 and α6. In contrast to cutaneous melanoma, in which α2 is well expressed in most lesions and α5 is expressed only in a small percentage of lesions, α2 expression was rare in uveal melanoma and α5 expression was found in all lesions. A major difference was observed with regard to the $\alpha v\beta 3$ vitronectin receptor. In contrast to cutaneous melanoma, in which $\alpha v\beta 3$ is expressed in advanced primary melanomas and metastases, $\alpha v\beta 3$ was not detected in any of the primary uveal melanomas, but all lesions strongly expressed $\alpha v\beta 5$.

Conclusions. Integrin expression in uveal melanoma cannot be correlated with cell type or invasiveness. In contrast to cutaneous melanoma, it seems that determination of the integrin expression profile is not suitable for categorizing uveal melanomas as less malignant and highly malignant lesions. Invest Ophthalmol Vis Sci. 1993;34:3635–3640.

In contrast to cutaneous melanomas, uveal melanomas metastasize primarily to the liver. This difference in biologic behavior cannot be explained on the basis of any known anatomic or physiologic factors.[1] Other sites of metastasis from uveal melanoma are the brain, lungs, bone marrow, lymph nodes, pericardium, skin, and organs of the gastrointestinal tract. Because the eye lacks lymphatics, uveal melanoma does not exhibit direct lymphatic spread, unlike cutaneous melanoma. In addition to direct hematogenous spread, uveal melanoma cells can invade the sclera and reach the orbital tissues, usually at sites where blood vessels, and possibly lymph vessels, pass through.

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Supported in part by the Dutch Cancer Society.
Submitted for publication December 8, 1992; accepted May 19, 1993.
Proprietary interest category: N.
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For malignant cells to detach from their primary location, attach to extracellular matrix components of the surrounding stroma, enter a blood vessel, and metastasize, their adhesive properties must change repeatedly.[2] Therefore, receptors mediating cell adhesion may be of great importance in metastasis.[3,4] Integrins, a superfamily of cell–surface receptors, participate in cell adhesion and migration.[5] These heterodimeric glycoproteins consist of an α-chain noncovalently linked to a β-chain, rendering ligand specificity. Integrins are divided into families on the basis of a common β-chain. So far, three families (β1, β2, β3) have been studied extensively, and five additional β-subunits were described recently.[5] In cutaneous melanocytic lesions, the level of expression of three integrin subunits (α2, α6, and β3) has been found to be correlated with tumor progression.[6–9]

Because uveal melanoma differs biologically and clinically from cutaneous melanoma, uveal melanoma may express a different pattern of integrins. In this

study, therefore, we investigated the expression pattern of a number of integrins in human uveal melanoma, using a panel of monoclonal antibodies (MoAbs) for immunohistochemical staining of α1-6, αv, β1, β4, αvβ3, and αvβ5 in frozen specimens.

MATERIALS AND METHODS

Specimens of 32 primary uveal melanomas and 4 uveal melanoma metastases were obtained from patients at University Hospital Nijmegen, Nijmegen; University Hospital Leiden, Leiden; University Hospital Rotterdam, Rotterdam; Academical Medical Center, Amsterdam; Foundation of Deventer Hospitals, Deventer, The Netherlands. Methods for securing human tissue were humane, included proper consent and approval, and complied with the Declaration of Helsinki.

Diagnosis of primary uveal melanomas was microscopically assessed on paraffin sections, discerning spindle ($N = 11$), mixed ($N = 14$), and epithelioid ($N = 7$) cell types. Scleral invasion was graded as follows: no invasion ($N = 3$), superficial invasion (less than 25% of scleral thickness) ($N = 11$), half invasion (50% of scleral thickness) ($N = 5$), deep invasion (75% of scleral thickness) ($N = 6$), and episcleral invasion ($N = 4$). Bruch's membrane was regarded as intact ($N = 9$) or ruptured ($N = 20$). In three primary uveal melanomas, invasion of the sclera or Bruch's membrane could not be determined.

Representative sections of all specimens were embedded in Tissue Tek OCT Compound (Ames Company, Division of Miles Laboratories, Elkhart, IN) and snap-frozen in liquid nitrogen. In a cryostat, 4-μm sections were cut serially at -25°C, mounted on uncoated slides, and stored at -80°C until use.

Integrin expression was detected by an indirect immunoperoxidase method with a panel of MoAbs listed in Table 1. In brief, sections were air-dried, fixed in acetone for 10 minutes, and incubated with the MoAbs at room temperature for 60 minutes. MoAbs were diluted in phosphate-buffered saline containing 1% bovine serum albumin, and optimal working dilutions were determined previously on positive controls. After the sections were rinsed with phosphate-buffered saline, the primary antibodies were detected with the use of peroxidase-based Vectastain Elite ABC system (Vector Laboratories, Burlingame, CA). This consisted of a biotinylated rabbit antirat IgG for MoAb GoH3 and a biotinylated horse antimouse IgG for all other MoAbs, followed by an avidin–biotin–peroxidase complex. As a substrate, 3-amino-9-ethyl-carbazole and hydrogen peroxide in acetate buffer (pH 4.85) were used. Finally, sections were counterstained with methyl green or hematoxylin and mounted with Kaiser's glycerol–gelatin (Merck, Darmstadt, Germany). Negative controls consisted of incubations replacing the primary antibodies with phosphate-buffered saline–bovine serum albumin.

The intensity of staining of the melanocytic cells was scored semiquantitatively as negative, positive, or strongly positive. The percentage of stained melanocytic cells was estimated as follows: 0%, 1% to 5%, 6% to 25%, 26% to 50%, 51% to 75%, and 76% to 100%. Slides were read independently by two observers. Discrepancies exceeding one percentage class were found in less than 10% of the cases. These cases were reevaluated jointly until a consensus was reached.

RESULTS

Staining results for the seven α-subunits and four β-subunits showed a consistent expression pattern among the 32 primary uveal melanomas (Fig. 1).

Uniformly positive staining was observed in all lesions when incubated with MoAbs against the common

TABLE 1. Monoclonal Antibodies

Integrin Subunit	*MoAb*	*Source and Reference*
α1	TS2/7	T-cell Science, Cambridge, MA[16]
α2	Gi14	Dr. Santoso, Giessen, Germany[17]
α2	A1.43	Dr. Bröcker, Würzburg, Germany[6,7]
α3	P1B5	Telios, San Diego, CA[18]
α4	HP2/1	Immunotech, Marseille, France[19]
α5	NKI-SAM1	Dr. Figdor, Amsterdam, The Netherlands[20]
α5	P1D6	Oncogene Science, Uniondale, NY[21]
α6	GoH3	Dr. Sonnenberg, Amsterdam, The Netherlands[22]
α6	MT78	Dr. Klein, Würzburg, Germany[23]
αv	NKI-M7	Dr. Figdor, Amsterdam, The Netherlands[24]
β1	4B4	Coulter, Hialeah, FL[25]
αvβ3	LM609	Dr. Cheresh, La Jolla, CA[26]
αvβ5	P1F6	Gibco, Gaithersburg, MD[27]
β4	3E1	Telios, San Diego, CA[28]

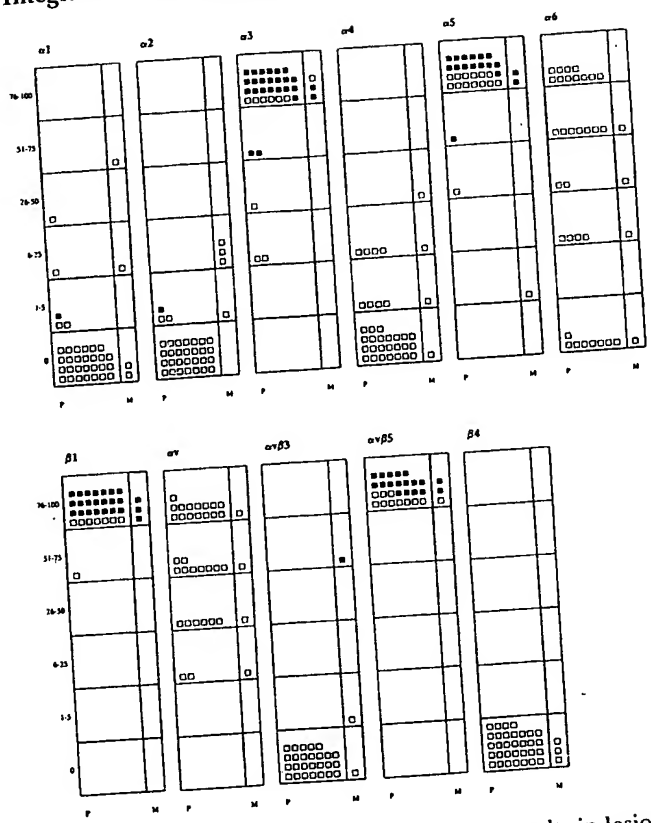

FIGURE 1. Percentage of cells with positive results in lesions stained with anti-integrin MoAbs. P: primary uveal melanoma; M: uveal melanoma metastasis. Numbers indicate percentage of cells with positive results. Staining intensity was scored as positive (□) or strongly positive (■).

β-subunit of the β1 integrins. A low proportion of lesions stained with MoAbs against the α1-(5 of 32, 16%), α2-(3 of 32, 9%), and α4-(8 of 32, 25%) subunits. When expression of these integrin subunits was detected, it usually was of moderate intensity, and a low percentage of tumor cells stained. A1.43 and Gi14 anti-α2 MoAbs showed identical staining patterns (not shown).

Expression of the α6-subunit was found in a majority of lesions (24 of 32, 75%), but considerable variability was observed in the number of positive tumor cells. That is, in 8 of 32 lesions, no positive tumor cells could be detected (Fig. 2c), whereas in 11 of 32 lesions, approximately all melanoma cells were stained but staining was usually cytoplasmic and of moderate intensity (Fig. 2d). A significant difference in reactivity was seen between GoH3 and MT78 anti-α6 MoAbs because the latter detected expression in only two lesions (not shown).

All lesions stained with MoAbs directed against α3 (Fig. 2a), α5 (Fig. 2b), and αv (not shown). Usually a high percentage of tumor cells within a given lesion stained strongly with these MoAbs, although staining with NKI-M7 anti-αv MoAbs was of moderate intensity. Expression of α5 always could be detected with NKI-SAM1 MoAbs, but few lesions stained with P1D6 MoAbs (not shown).

The β4-subunit could not be detected on melanoma cells in any of the lesions, but staining of blood vessels was observed with 3E1 anti-β4 MoAbs (not shown). No expression of αvβ3 was observed in any of the primary lesions, whereas staining of blood vessels was observed with LM609 anti-αvβ3 MoAbs (Fig. 2e). In contrast, all lesions strongly expressed αvβ5 (Fig. 2f).

Similar to primary uveal melanoma, all metastatic lesions expressed the β1-subunit and none of them expressed the β4-subunit (Fig. 1). Furthermore, all metastatic lesions expressed α3, α5, and αv and three of four expressed α6. In contrast to primary uveal melanoma, all metastatic lesions showed α2 and three of four showed α4 expression, but a low percentage of cells within a given lesion stained and the staining was of moderate intensity. Again, similar to the results for primary uveal melanoma, 76% to 100% of the cells in all three uveal melanoma metastases expressed αvβ5. In two lesions strong staining was observed, and in one lesion staining was of moderate intensity (Fig. 2h); however, expression of αvβ3, which was not detected in any of the primary lesions, could be detected in most cells of one of the metastatic lesions (Fig. 2g) and in some cells of another metastatic lesion.

Some sections from one of the metastatic lesions showed mainly necrotic tumor tissue, and no additional material was available. Therefore, staining with anti-α3, -α5, -β1, -αvβ3, -αvβ5, and -β4 MoAbs could be investigated in only three metastatic lesions.

Within our series of primary tumors, neither the cell type nor the invasion of sclera or Bruch's membrane correlated with an expression preference for any of the integrin subunits.

DISCUSSION

Integrins in cutaneous melanoma have been the subject of a number of studies investigating the pathogenesis of metastasis.[10] Clinical and biologic differences between cutaneous and uveal melanoma led us to question whether different integrin expression patterns could be found for these tumors. To the best of our knowledge, this study is the first to investigate the expression pattern of a large number of integrins in human uveal melanoma. In our panel of primary uveal melanomas, all cell types and stages are represented. Because patients with metastases of uveal melanoma usually die at home, it is difficult to collect a set of metastases. Nevertheless, we gathered four uveal melanoma metastases, but none from the liver. Due to this low number, no significant conclusions can be drawn regarding integrin expression in uveal melanoma me-

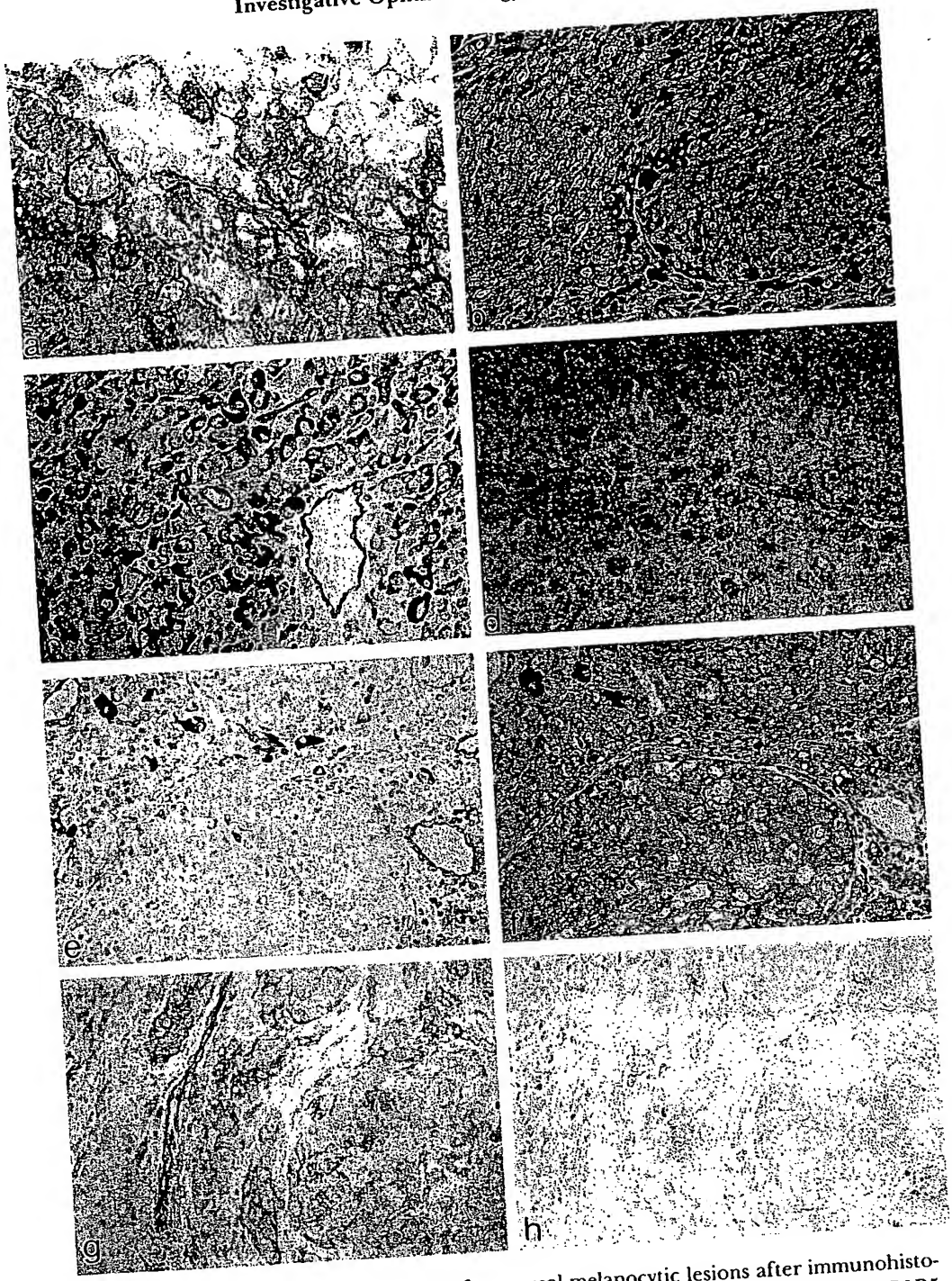

FIGURE 2. Photomicrographs of sections from uveal melanocytic lesions after immunohistochemical staining with anti-integrin MoAbs. (A) Primary uveal melanoma stained with P1B5 anti-α3 MoAbs. (B) Primary uveal melanoma stained with NKI-SAM1 anti-α5 MoAbs. (C, D) Primary uveal melanoma lesions stained with GoH3 anti-α6 MoAbs. Notice that (C) contains no tumor cells with positive results (vessel walls stain strongly), whereas in (D) approximately 100% of the melanoma cells had positive results. (E, F) Primary uveal melanoma stained with (E) LM609 anti-$\alpha v\beta 3$ MoAbs or (F) P1F6 anti-$\alpha v\beta 5$ MoAbs. Identical regions in sequential sections are shown. (G, H) Uveal melanoma metastasis stained with (G) LM609 anti-$\alpha v\beta 3$ MoAbs or (H) P1F6 anti-$\alpha v\beta 5$ MoAbs. Identical regions in sequential sections are shown. (A, $\times 400$; B, $\times 200$; C, $\times 200$; D, $\times 400$; E, F, G, H, $\times 200$.)

tastasis. The results of our survey have no diagnostic or prognostic implications, but they may enhance the understanding of biologic processes with respect to uveal melanoma.

Comparison of integrin expression in this study and studies on cutaneous melanoma indicates some differences. In uveal melanoma, we find that all lesions express $\alpha 5$ but only a few have positive results for $\alpha 2$, whereas most cutaneous melanoma lesions express the $\alpha 2$-subunit, and $\alpha 5$ can be detected only in approximately 20% of them.[8] Furthermore, although no $\beta 4$ can be detected in uveal melanomas, a small number of cutaneous melanomas express this subunit.[9] The fact that only few lesions have positive results for $\alpha 4$, whereas $\alpha 3$, $\alpha 6$, and αv can be detected in most lesions, is similar to the findings in cutaneous melanoma.[8,9] The different staining profile with different MoAbs against the same integrin subunit, as seen with MoAbs against $\alpha 5$ and $\alpha 6$, most probably results from differences in the avidity of the MoAbs or masking of specific epitopes.

We recently confirmed and extended the data on integrin expression in cutaneous melanoma in a large set of 115 melanocytic lesions.[10] The most striking difference between cutaneous and uveal melanoma concerns the $\alpha v\beta 3$ vitronectin receptor. In cutaneous melanoma, $\alpha v\beta 3$ is expressed in metastases and vertical growth phase primary lesions, but not in nevi or the relatively benign radial growth phase primary lesions.[8] In this study on uveal melanoma, no $\alpha v\beta 3$ expression could be detected in any of the primary lesions, including those that may have metastatic potential, such as those of the mixed and epithelioid cell types in which Bruch's membrane was broken and sclera invaded. This indicates that the αv-subunit in uveal melanoma combines with an alternative subunit. We showed that $\alpha v\beta 5$ is the vitronectin receptor expressed in primary uveal melanoma. In uveal melanoma metastasis, both $\alpha v\beta 3$ and $\alpha v\beta 5$ may be expressed. It is known that αv can combine with $\beta 1$, $\beta 3$, $\beta 5$, $\beta 6$, or $\beta 8$.[5] Because no MoAbs recognizing the $\alpha v\beta 1$, $\alpha v\beta 6$, or $\alpha v\beta 8$ complexes were available, we cannot exclude the possibility that any of these integrins are present in uveal melanoma as well. Integrin $\alpha v\beta 5$ appears to be restricted to vitronectin in its ligand binding specificity, whereas $\alpha v\beta 3$ interacts with multiple ligands.[12] Furthermore, $\alpha v\beta 3$ and $\alpha v\beta 5$ promote distinct cellular responses to vitronectin in vitro.[13]

These findings indicate that the difference in expression of αv integrins between cutaneous and uveal melanoma may lead to a different biologic behavior. Because αv-integrins are thought to play a role in proliferation[14] and invasion[15] of melanoma cells, the lack of $\alpha v\beta 3$ in primary uveal melanoma may have consequences for its growth and metastatic behavior. The fact that histologic differences in cell type and scleral and Bruch's membrane invasion cannot be correlated with a difference in integrin expression suggests that determination of the integrin expression profile is not suitable for subdividing uveal melanomas into less malignant and highly malignant lesions. In contrast, this seems possible in cutaneous melanomas.[8,9,10]

In conclusion, we found that integrin expression in human uveal melanoma is not correlated with cell type or invasiveness. Furthermore, we found that the $\alpha v\beta 3$ vitronectin receptor, which is associated with cutaneous melanoma progression, is not expressed in primary uveal melanoma. In uveal melanoma, we showed that the αv-subunit combines with $\beta 5$.

Key Words

uveal melanoma, metastasis, integrins, immunohistochemistry, vitronectin receptor

Acknowledgments

The authors thank Drs. E. B. Bröcker, D. A. Cheresh, C. G. Figdor, C. E. Klein, S. Santoso, and A. Sonnenberg for generously providing the MoAbs. They also thank Drs. C. Mooy, J. J. Weening, and E. F. Weltevreden for providing some of the lesions, and José Aldeweireldt for expert technical assistance.

References

1. Einhorn LH, Burgess MA, Vallejos C, et al. Advanced metastatic malignant melanoma: Prognostic correlations and response to treatment in 426 patients. *Cancer Res.* 1974;34:1995–2004.
2. Poste G, Fidler Y. The pathogenesis of cancer metastasis. *Nature.* 1980;283:139–146.
3. Liotta L. Tumor invasion and metastasis: Role of the extracellular matrix: Rhoads Memorial Award Lecture. *Cancer Res.* 1987;46:1–7.
4. Ruoslahti E, Giancotti FG. Integrins and tumor cell dissemination. *Cancer Cells.* 1989;1:119–126.
5. Hynes RO. Integrins: Versatility, modulation and signaling in cell adhesion. *Cell.* 1992;69:11–25.
6. Bröcker EB, Suter L, Brüggen J, Ruiter DJ, Macher E, Sorg C. Phenotypic dynamics of tumor progression in human malignant melanoma. *Int J Cancer.* 1985;36: 29–35.
7. Klein CE, Steinmeyer T, Kaufmann D, Weber L, Bröcker EB. Identification of a melanoma progression antigen as integrin VLA-2. *J Invest Dermatol.* 1991;96:281–284.
8. Albelda SM, Mette SA, Elder DE, et al. Integrin distribution in malignant melanoma: Association of the $\beta 3$ subunit with tumor progression. *Cancer Res.* 1990;50: 6757–6764.
9. Natali PG, Nicotra MR, Cavaliere R, Giannarelli D, Bigotti A. Tumor progression in malignant melanoma is associated with changes in $\alpha 6\beta 1$ laminin receptor. *Int J Cancer.* 1991;47:168–172.
10. Danen EHJ, ten Berge PJM, van Muijen GNP, Bröcker E-B, Ruiter DJ. Emergence of $\alpha 5\beta 1$ fibronec-

tin- and αvβ3-vitronectin receptor in melanocytic tumor progression *Histopathology*. In press.
11. Hart IR, Birch M, Marshall JF. Cell adhesion receptor expression during melanoma progression and metastasis. *Cancer Metastasis Rev.* 1991;10:115–128.
12. Smith JW, Vestal DJ, Irwin SV, Burke TA, Cheresh DA. Purification and functional characterization of integrin αvβ5: An adhesion receptor for vitronectin. *J Biol Chem.* 1990;265:11008–11013.
13. Leavesley DI, Ferguson GD, Wayner EA, Cheresh DA. Requirement of the integrin β3 subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen. *J Cell Biol.* 1992;117:1101–1107.
14. Felding-Habermann B, Mueller BM, Romerdahl CA, Cheresh DA. Involvement of integrin αv gene expression in human melanoma tumorigenicity. *J Clin Invest.* 1992;89:2018–2022.
15. Seftor REB, Seftor EA, Gehlsen KR, et al. Role of the αvβ3 integrin in human melanoma cell invasion. *Proc Natl Acad Sci U S A.* 1992;89:1557–1561.
16. Hemler ME, Sanchez-Madrid F, Flotte TJ, et al. Glycoproteins of 210,000 and 130,000 m.w. on activated T-cells: Cell distribution and antigenic relation to components on resting cells and T-cell lines. *J Immunol.* 1984;132:3011–3018.
17. Santoso S, Kiefel V, Müller-Eckhardt C. Immunochemical characterization of the new platelet alloantigen system. *Br J Haematol.* 1989;72:191–198.
18. Wayner EA, Carter WG. Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique α and common β subunits. *J Cell Biol.* 1987;105:1873–1884.
19. Sanchez-Madrid F, De Landazuri MO, Morago G, Cebrian M, Acevedo A, Bernabeu C. VLA-4: A novel polypeptide association within the VLA molecular complex: Cell distribution and biochemical characterization. *Eur J Immunol.* 1986;16:1343–1349.
20. Van de Wiel-van Kemenade E, Van Kooyk Y, De Boer AJ, et al. Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the β subunit of VLA. *J Cell Biol.* 1992;117:461–470.
21. Wayner EA, Carter WG, Piotrowicz RS, Kunicki TJ. The function of multiple extracellular matrix receptors in mediating adhesion to extracellular matrix: Preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion of fibronectin and react with platelet glycoproteins IcIIa. *J Cell Biol.* 1988;107:1881–1891.
22. Sonnenberg A, Janssen H, Hogervorst F, Calafat J, Hilgers J. A complex of platelet glycoproteins Ic and IIa identified by a rat monoclonal antibody. *J Biol Chem.* 1987;264:13745–13750.
23. Klein CE, Steinmeyer T, Mattes J, Kaufmann R, Weber L. Integrins of normal human epidermis: Differential expression, synthesis and molecular structure. *Br J Dermatol.* 1990;123:171–178.
24. De Vries JE, Keizer GD, te Velde AA, et al. Characterization of melanoma-associated surface antigens involved in the adhesion and motility of human melanoma cells. *Int J Cancer.* 1986;38:465–473.
25. Morimoto C, Letvin NL, Boyd AW, et al. The isolation and characterization of the human helper inducer T cell subset. *J Immunol.* 1985;134:3762–3769.
26. Cheresh DA, Harper JR. Arg-Gly-Asp recognition by a cell adhesion receptor requires its 130 kDa α-subunit. *J Biol Chem.* 1987;262:1434–1437.
27. Wayner EA, Orlando RA, Cheresh DA. Integrins αvβ3 and αvβ5 contribute to cell attachment to vitronectin but differentially distribute on the cell surface. *J Cell Biol.* 1991;113:919–929.
28. Hessle H, Sakai LY, Hollister DW, Burgeson RE, Engvall E. Basement membrane diversity detected by monoclonal antibodies. *Differentiation.* 1984;26:49–54.

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Influence of Autonomic Neurotransmitters on Human Uveal Melanocytes in vitro

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(*Received Seattle 23 November 1999 and accepted in revised form 15 March 2000*)

The influence of autonomic neurotransmitters on the growth and melanogenesis of cultured uveal melanocytes was studied. Uveal melanocytes were cultured with medium supplemented with cAMP elevating agents and basic fibroblast growth factor (complete medium). The cells were plated into multiple well plates, and various concentrations of adrenergic and cholinergic agents were added to the media (complete medium or various deleted media). After 6 days, the cells were detached for cell counting and melanin measurement and compared to controls. Epinephrine, isoproterenol, salbutamol and metaproterenol (adrenergic agonists that can activate β_2-adrenoceptors) substantially stimulated growth and melanogenesis of cultured uveal melanocytes in cAMP-deleted medium. Methoxamine, clonidine, prenalterol and D-7114 (adrenergic agonists that do not activate β_2-adrenoceptors) showed no effect under similar experimental conditions. Muscarine (a cholinergic agonist) inhibited the growth and melanogenesis of uveal melanocytes in complete medium. It indicates that adrenergic agents (β_2-adrenoceptor agonists) stimulate growth and melanogenesis in uveal melanocytes, while cholinergic agonist has an inhibitory effect. This effect appears to involve the cAMP second messenger system. These studies suggest that homeostasis of the uveal melanocytes may be maintained, in part, by regulating the autonomic nervous system in vivo.

Key words: adrenergic agonists; autonomic nerves; cholinergic agonists; neurotransmitters; uveal melanocytes; melanin; Horner's syndrome.

1. Introduction

Iris pigmentation results mainly from the presence of uveal melanocytes (UM) and the iris pigment epithelium (IPE). Individual differences in iris color are determined primarily by variable melanin content in iridial melanocytes (Eagle, 1988; Imesch et al., 1996; Wilkerson et al., 1996). Iris depigmentation occurs in sympathetic nerve paralysis (Horner's syndrome), and interruption of the sympathetic pathway in experimental animals also causes iris depigmentation and decreases tyrosinase activity in the iris and choroid (Laties, 1974). Ultrastructural and neurohistochemical studies in human and various experimental animals suggest close contact between uveal melanocytes and both adrenergic and cholinergic nerve termini (Wolter, 1960; Feeney and Hogan, 1961; Ehiger, 1966; Hogan, Alvarado and Weddell, 1971; Laties, 1972, 1974; Ringvold, 1975; Mukuno and Witmer, 1977; Tamm et al., 1997). These findings indicate the presence of innervation of UM. It is possible that the growth and melanogenesis of uveal melanocytes may be regulated by the autonomic nervous system. However, the effects of adrenergic and cholinergic stimulation on the UM and the types of neurotransmitter receptors involved have not been investigated thoroughly.

We have developed methods for isolation and culture of human UM. The UM grow well and can divide 35–50 times in vitro (Hu, McCormick and Ritch, 1993a; Hu et al., 1993b). Cultured UM express tyrosinase activity and produce melanin in vitro. The melanin content of cultured UM correlates with the melanin content in vivo (Hu et al., 1995, 1997). Therefore, these cells can be a good model for studying the cell biology of UM. In the present study, we investigated the effects of various neurotransmitters and receptor selective adrenoceptor agonists (Table I) on the growth and melanogenesis of cultured UM.

2. Materials and Methods

Reagents

F12 nutrient mixture (F12 medium), fetal bovine serum (FBS), geneticin, L-glutamine, gentamicin, trypsin solution, and trypsin-ethylene diaminetetraacetic acid (trypsin-EDTA) solution were obtained from GIBCO (New York, NY, U.S.A.). 12-O-tetradecanoyl-phorbol-13-acetate (TPA), synthetic melanin by oxidation of tyrosine with hydrogen peroxide, cholera

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TABLE I

Various adrenergic agonists tested in the present study

	Receptors	2nd Messenger
Epinephrine	α and β	Phospholipase C, Ca^{++}, cAMP
Phenylephrine	α_1 and β	Phospholipase C, Ca^{++}, cAMP
Methoxamine	α_1	Phospholipase C, Ca^{++}
Clonidine	$\alpha_2 > \alpha_1$	Phospholipase C, Ca^{++}
Isoproterenol	β	cAMP
Prenalterol	β1	cAMP
Salbutamol	β2	cAMP
Metaproterenol	β2	cAMP
D-7114	β3	cAMP

Ref.: Brittain et al. (1968); Cantacuzene et al. (1979); Nimit et al. (1980); Scarpace and Abrass (1982); Cook, Richardson and Barnett (1984); Minneman (1988); Emorine et al. (1991); Holloway et al. (1991); Lanier et al. (1996).

toxin, isobutylmethylxanthine (IBMX), epinephrine, phenylephrine, methoxamine, clonidine, metaproterenol and isoproterenol were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Human recombinant bFGF was obtained from Promega (Madison, WI, U.S.A.). Prenalterol, salbutamol and D-7114 were obtained from Astra (Worcester, MA, U.S.A.), Glaxo (Ware, U.K.) and ICI (Macclesfield, U.K.), respectively.

Cell Culture

Human UM were isolated from adult donor eyes as previously described (Hu et al., 1993b). The isolated UM were cultured with FIC medium, which is F12 medium supplemented with 10% FBS, 2 mM glutamine, 20 ng ml^{-1} bFGF, 0·1 mM IBMX, 10 ng ml^{-1} cholera toxin, and 50 μg ml^{-1} gentamicin. After reaching confluence, the UM were detached using trypsin–EDTA solution, diluted 1:3–1:6, and subcultured.

Experiments

The UM used in this study consisted of four cell lines, two isolated from the iris and two from the choroid. These cells had been in culture for no longer than 2 months and had been passaged three to six times at a dilution of 1:3–1:4. The purity of the cell lines was demonstrated by immunocytochemical methods. UM displayed S-100 antigen but no cytokeratin antigen, the pigment epithelial cells displayed both antigens, and the fibroblasts displayed neither of these antigens (Hu et al., 1993b).

The UM were plated into 12 well plates with FIC medium at a density of 5×10^4 cells per well. After 24 hr, the FIC medium was replaced with 1·0 ml of the test media. The media were replaced every 3 days, and the cells were cultured for 6 days. After 6 days, the cells were detached by trypsin and counted using a hemocytometer. Detached cells were treated with sodium hydroxide (1 M) to extract the melanin. The amount of melanin was then measured using a spectrophotometer at 475 nm and compared with a standard curve to determine the amount of melanin present (expressed as μg per well).

Effect of adrenergic agonists. The base medium used was cAMP-deleted medium, which consisted of F12 medium with 10% FBS and 20 ng ml^{-1} bFGF. Various adrenergic agonists (epinephrine, isoproterenol, phenylephrine, clonidine, methoxamine, prenalterol, salbutamol, D-7114 and metaproterenol at a concentration of 10^{-5} M) were added to test their effects. Phenylephrine, clonidine and methoxamine were tested both in cAMP-deleted medium and bFGF/TPA-deleted medium (medium with serum, cAMP-elevating agents but without bFGF and TPA). Each was tested in triplicate. UM cultured with cAMP-deleted medium, bFGF/TPA-deleted medium and complete medium (FIC medium) were used as negative and positive controls, respectively.

Effect of isoproterenol in various media. The β-adrenoceptor agonist isoproterenol was selected as a model for further studies on the effect of adrenergic agonist on UM in various media. The base media used included serum-deleted medium (medium with FIC, but without serum), cAMP-deleted medium, bFGF/TPA-deleted medium, and complete medium (FIC medium). Isoproterenol (10^{-5} M) was added to test its effect on cell growth and melanogenesis in various types of media. Each was tested in triplicate. UM cultured with complete medium (FIC medium) and various deleted media were used as negative and positive controls, respectively.

Dose-dependent effects of isoproterenol. The base medium used was cAMP-deleted medium. Isoproterenol was added in graded concentrations (from 10^{-10} to 10^{-5} M), each concentration was tested in triplicate. UM cultured with cAMP-deleted medium and complete medium (FIC medium) were used as negative and positive controls, respectively.

Effects of phenylephrine at different concentrations in various media. Phenylephrine stimulated cell growth and melanogenesis in cAMP-deleted medium at a concentration of 10^{-5} M. Phenylephrine activates both α- and β-adrenoceptors. In order to elucidate which receptor phenylephrine activated in UM, phenylephrine (10^{-7}–10^{-5} M) was added to UM cultured with cAMP-deleted medium, bFGF/TPA-deleted medium and serum-deleted medium. Each concentration was tested in triplicate. UM cultured with complete medium (FIC medium) and various deleted media were used as negative and positive controls, respectively.

Effects of cholinergic agonist. The base media used were serum-deleted medium and complete medium.

Muscarine was added at various concentrations from 10^{-6} to 10^{-4} M to test its effects. Each was tested in triplicate. UM cultured with complete medium (FIC medium) and serum-deleted medium were used as controls.

Effect of deletion of adrenergic agonist. The UM were plated into 6 well plates at a cell density of 2×10^4 and cultured with cAMP-deleted medium supplemented with isoproterenol (10^{-5} M). Three days later, isoproterenol was deleted from the culture medium in 21 wells of UM. Cells were detached from three wells for cell counting and melanin measurement every 3 days for 3 weeks. UM cultured with isoproterenol supplemented medium were used as positive controls.

Some tested neurotransmitters are not stable and are easily oxidized, therefore an antioxidant, ascorbic acid (10^{-5} M), was added to all culture media to protect the tested substances.

Student's *t*-test was used to assess statistical significance.

3. Results

UM cultured with cAMP-deleted medium grew slowly and showed a decrease of melanin content/culture. Addition of epinephrine, phenylephrine, isoproterenol, salbutanol and metaproterenol at a concentration of 10^{-5} M resulted in significant stimulation of growth and an increase in the melanin content/culture ($P < 0.01$) (Table II). Methoxamine, clonidine, prenalterol and D-7114 at a concentration of 10^{-5} M showed no effect on cell growth or melanin content (Table II). Phenylephrine, clonidine and methoxamine at a concentration of 10^{-5} M showed no effects in bFGF/TPA-deleted medium (data not shown).

UM cultured with bFGF/TPA-deleted medium and serum-deleted medium grew slowly or did not grow, and melanin content/culture decreased in cells cultured with serum-deleted medium. Isoproterenol (10^{-5} M) stimulated cell growth significantly in cells cultured with cAMP-deleted medium and to a lesser degree in bFGF/TPA-deleted medium and complete medium, but not in serum-deleted medium, indicating that isoproterenol mainly activated the cAMP system [Fig. 1(A)].

Isoproterenol stimulated growth and melanogenesis in a dose-dependent manner at concentrations ranging from 10^{-10} to 10^{-5} M [Fig. 1(B)]. The difference in melanin content/culture and cell counts between the negative control and the test groups were statistically significant ($0.05 > P > 0.01$ at concentrations of 10^{-8}–10^{-9} M and $P < 0.01$ at concentrations of 10^{-7}–10^{-5} M).

Phenylephrine stimulated growth and melanogenesis of UM only in cells cultured with cAMP-deleted medium and at a concentration of 10^{-5} M ($P < 0.01$). Phenylephrine did not affect the cell number and melanin content of UM in bFGF/TPA-deleted medium and serum-deleted medium (10^{-6}–10^{-5} M) and in cAMP-deleted medium at a concentration of 10^{-6} M [Fig. 2(A)]. These results indicate that phenylephrine only activates the cAMP system at a high concentration. Therefore, it is possible that the effect of phenylephrine on UM is related to activation of β-adrenoceptors of UM, since phenylephrine is well known to possess weak β-adrenoceptor agonist properties (Cantacuzene et al., 1979; Nimit et al., 1980).

Muscarine inhibited the growth and melanogenesis of UM cultured with complete medium and serum-deleted medium [Fig. 2(B)]. The differences of melanin content/culture and cell counts between the control and test groups were statistically significant (cell

TABLE II

Effect of various adrenergic agonists on the growth and melanogenesis of cultured uveal melanocytes

Group	Neurotransmitters	Cell number	Melanin per culture (per cell)
Negative control (deleted medium)	–	100 ± 7%	100 ± 11·7% (100%)
Non-selective adrenergic agonist	Epinéphrine	294 ± 20%*	222 ± 21·4%* (75·8%)
α-adrenoceptor agonist:			
(α_1- and β)	Phenylephrine	149 ± 13%*	127 ± 16·3% (85·2%)
(α_1)	Methoxamine	102 ± 9%	100 ± 12·1% (98·0%)
(α_2)	Clonidine	98 ± 6%	98 ± 10·8% (100%)
Non-selective β-adrenoceptor agonist	Isoproterenol	305 ± 25%*	214 ± 19·4%* (70·2%)
Selective β-adrenoceptor agonist:			
(β_1)	Prenalterol	120 ± 8%	106 ± 11·7% (88·3%)
(β_2)	Metaproterenol	311 ± 21%*	216 ± 32·6%* (69·5%)
(β_2)	Salbutamol	298 ± 15%	211 ± 21·4%* (70·8%)
(β_3)	D-7114	103 ± 6%	100 ± 10·3% (97·1%)
Positive control (FIC medium)	–	321 ± 22%*	308 ± 35·3%* (95·9%)

Cells were plated into 12 well plates and cultured in cAMP-deleted medium. Various adrenergic agonists (10^{-5} M) were added. Each was tested in triplicate. Cells cultured with cAMP-deleted medium or complete medium were used as negative and positive controls, respectively. After 6 days, the cells were counted, and melanin content was measured. Cell number and melanin content were expressed as a percentage of the negative control (mean ± S.D.). Melanin content per culture, not per cell, was used as the main parameter for evaluating melanogenesis (see Discussion). *$P < 0.01$.

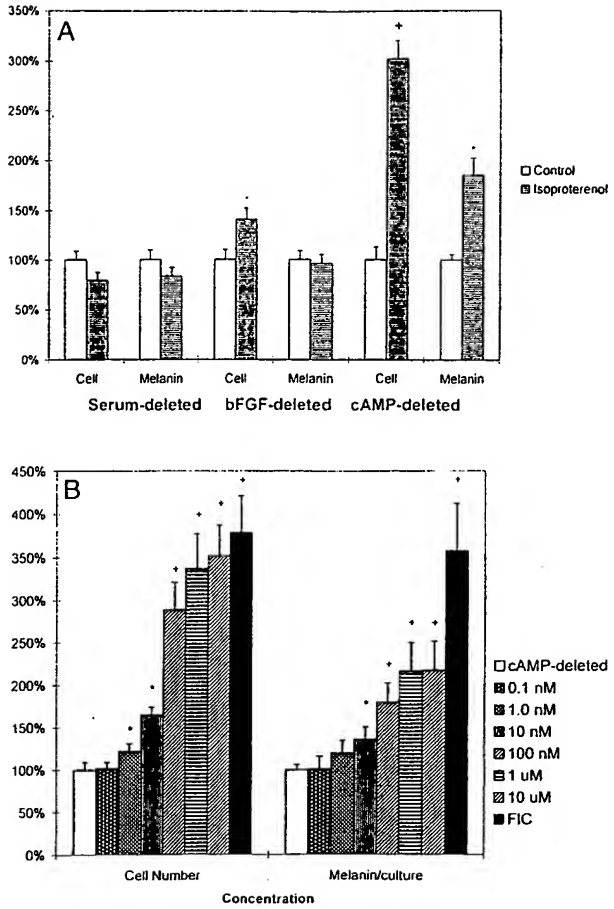

FIG. 1. Effect of isoproterenol on the growth and melanogenesis of uveal melanocytes in different culture media (A) or different concentrations (B). Cells were plated into 12 well plates and cultured in serum-deleted, bFGF/TPA deleted and cAMP-deleted media (A) or in cAMP-deleted media (B). Isoproterenol [at 10^{-5} M in (A) and at 10^{-10}–10^{-5} M in (B)] was added to test its effect. Cells cultured with various deleted media were used as negative controls. Each was tested in triplicate. The media were replaced every 3 days. After 6 days, the cells were detached and counted, melanin was extracted and measured. Both numbers of cell and melanin contents per well were expressed as percentages of the negative control (mean ± S.D.). *$0{\cdot}05 > P > 0{\cdot}01$; +$P < 0{\cdot}01$.

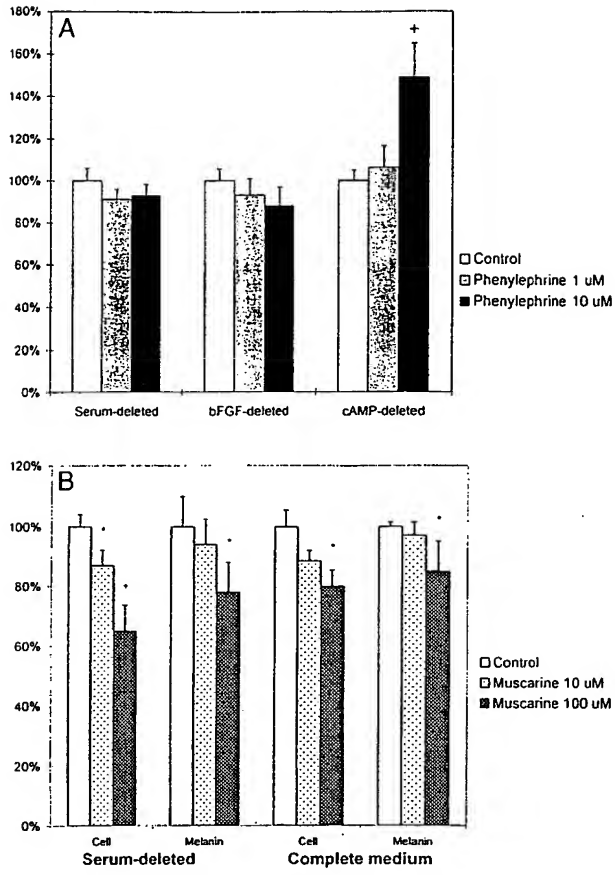

FIG. 2. Effects of phenylephrine (A) or muscarine (B) at different concentrations on the growth and melanogenesis of uveal melanocytes. Cells were plated into 12 well plates and cultured in serum-deleted, bFGF/TPA deleted and cAMP-deleted media (A) or serum-deleted medium and complete medium (B). Phenylephrine [10^{-6}–10^{-5} M, (A)] or muscarine [10^{-5}–10^{-4} M, (B)] was added to test their effect. Cells cultured with various deleted media (A) or serum-deleted medium and complete medium (B) were used as controls. Each was tested in triplicate. The media were replaced every 3 days. After 6 days, the cells were detached and counted, melanin was extracted and measured. Both numbers of cell and melanin contents per well were expressed as percentages of the control (mean ± S.D.). *$0{\cdot}05 > P > 0{\cdot}01$; +$P < 0{\cdot}01$.

number: $0{\cdot}05 > P > 0{\cdot}01$ at concentrations of 10^{-4} M in complete medium, at 10^{-5} M in serum-deleted medium, and $P < 0{\cdot}01$ at concentrations of 10^{-4} M in serum-deleted medium; melanin content/culture: $0{\cdot}05 > P > 0{\cdot}01$ at concentration of 10^{-4} M in complete medium and serum-deleted medium).

UM cultured with cAMP-deleted medium supplemented with 10^{-5} M isoproterenol grew well. After deletion of isoproterenol, UM still grew and produced melanin for 3 days, thereafter, cells grew slowly. Seven days after the deletion of isoproterenol, the UM ceased to grow, became degenerated and detached from the well, the number of cells and melanin content per well decreased continuously. The number of UM decreased to 25% of total seeded cells after 21 days of deletion of isoproterenol. In contrast, the UM cultured with medium supplemented with isoproterenol grew well and produced melanin during the 3 week period (Fig. 3).

All data mentioned above were based on the study of a melanocyte cell line from iris. The effects of neurotransmitters on growth and melanogenesis of cultured UM were consistent in all three other cell lines.

4. Discussion

There are two types of melanin-synthesizing cells in the iris: the IPE and iridal melanocytes (Hogan et al., 1971; Mund, Rodrigues and Fine, 1972; Zinn et al., 1973; Imesch et al., 1996; Wilkerson et al., 1996). The IPE is located at the posterior surface of

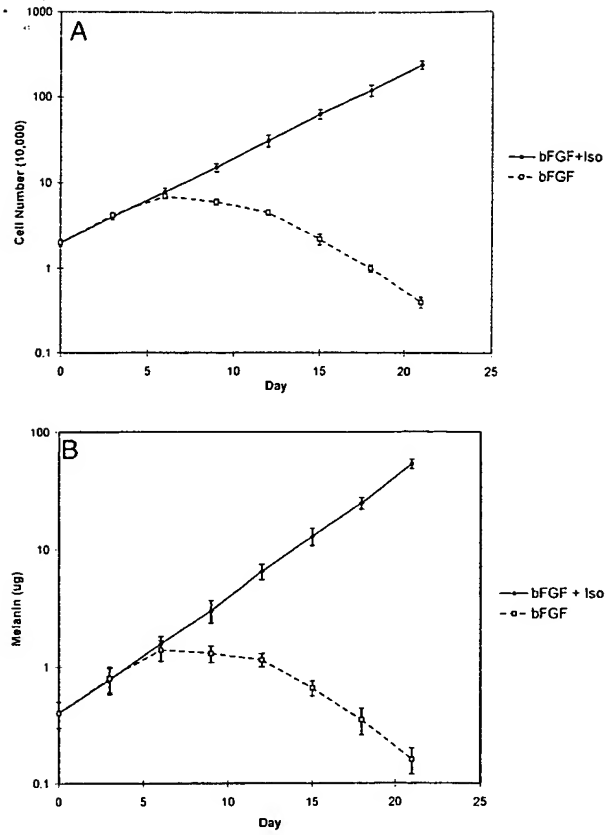

FIG. 3. Effect of deletion of isoproterenol on the growth and melanogenesis of uveal melanocytes. Uveal melanocytes were plated into 6 well plates and cultured with cAMP-deleted medium supplemented with isoproterenol (10^{-5} M), 3 days later, isoproterenol was deleted from the culture medium in 21 wells (dashed lines). Cells were detached from three wells for cell counting and melanin measurement every 3 days for 3 weeks. Cells cultured with isoproterenol supplemented medium were used as positive controls (solid line). Cell number was expressed as 1×10^4 (mean $\pm$ S.D.) (A). Melanin content was expressed as μg per well (mean $\pm$ S.D.) (B).

the iris and is derived from the neuroectoderm. Adult human IPE do not produce melanin in vitro (Hu et al., 1992). The melanin in IPE does not vary significantly between irides of different colors. Therefore, the IPE is believed to play only a minor role in variations in iris color (Eagle, 1988; Imesch et al., 1996; Wilkerson et al., 1996).

The melanocytes are derived from the neural crest, are located in the stroma of the iris, and tend to cluster near the anterior border of the stroma (Hogan et al., 1971; Zinn et al., 1973; Tamm et al., 1997). Adult human UM do produce melanin in vitro (Hu et al., 1995). The melanin content of melanocytes, which varies in different colored irides (both in vivo and in vitro), is the most important factor in determining iris color (Eagle, 1988; Hu et al., 1995; Imesch et al., 1996; Wilkerson et al., 1996).

It has been long recognized that hypopigmentation (congenital cases) or depigmentation (acquired cases) of the iris on the affected side occur in patients with paralysis of the cervical sympathetic nerve (Horner's syndrome). The mechanism of iris depigmentation in Horner's syndrome has been obscure. There are different hypotheses involving sympathetic nerves: these include neurotrophic effects, control of migration, or the supply of noradrenaline to UM for use as a substrate for conversion to a melanin akin to neuromelanin (Laties, 1972, 1974). Electron microscopy studies of the iris from a Horner's syndrome patient revealed depletion of UM and absence of sympathetic nerve fibers. In contrast to the UM, the IPE still remained unaffected (McCartney et al., 1992).

In experimental animals, removing the superior cervical ganglion or section of the sympathetic nerve trunk below the ganglion in rabbits or cats leads to iris depigmentation and decreased tyrosinase activity in the iris and choroid (Laties, 1972, 1974). Topical administration of adrenergic antagonist in newborn rabbits also causes iris depigmentation (Odin and O'Donnell, 1982).

Neurohistochemical studies, anatomic and ultrastructure studies in various experimental animals (cat, rabbit, rat, baboon and monkey) and humans revealed a close relationship between adrenergic nerve termini, postganglionic cholinergic nerve termini and UM (Wolter, 1960; Feeney and Hogan, 1961; Ehiger, 1966; Hogan et al., 1971; Laties, 1972, 1974; Ringvold, 1975; Mukuno and Witmer, 1977; Tamm et al., 1997). These observations point to a dual autonomic innervation of UM. However, the effects of adrenergic and cholinergic nerve stimulation on melanogensis and growth of UM have not been studied, and little is known about the exact receptors and second messenger system involved.

During the past decade, we have developed methods for isolation and cultivation of human UM, collected many cell lines and studied factors regulating melanogenesis and growth of UM in vitro (Hu et al., 1993b, 1993a, 1995, 1997). We found that growth and melanogenesis of cultured UM were controlled by at least three second messenger systems. cAMP elevating agents and protein kinase C activators (TPA) stimulate melanogenesis and growth of cultured UM. Tyrosine kinase activators (bFGF) stimulate growth but not melanogenesis of cultured UM. Serum, cAMP-elevating agents, bFGF or TPA, are essential for growth, melanogenesis and survival of cultured UM. Deprivation of any factors leads to a decrease of melanin content and or cell numbers (Hu et al., 1993a, 1997). Based on our previous work, we have developed a deletion and substitution model for studying the effects and mechanism of various substances on UM. One of the known factors is deleted from the complete medium, and a test substance is added to the deleted media. If the UM grow and differentiate well, then we suppose that the test substance may have a similar mode of action as the agent deleted (Hu et al., 1993a, 1997).

In our previous studies, three parameters (melanin content per cell, melanin content per culture and tyrosinase activity) were used to evaluate the effect of

various substances on melanogenesis in cultured UM (Hu et al., 1995, 1997). An important concept for consideration is which parameter is most meaningful to evaluate melanogenesis of UM in vitro. Tyrosinase activity is an important factor, but not the sole factor in determining the rate of melanin production (Prota, 1993). The color of the iris is determined by melanin content rather than tyrosinase activity (Hu et al., 1995). Measurement of melanin content is more direct and meaningful than that of tryosinase activity. Melanin content per culture is more important than melanin content per cell for two reasons. First, melanin content per cell is affected both by melanin production rate and the growth rate. In stationary cells, the melanin produced accumulates in the cell and results in a rapid increase in the melanin content per cell. In growing cells, the melanin is diluted to daughter cells during division and results in a marked decrease of melanin per cell (Hu et al., 1997). Furthermore, from the clinical view point, iris pigmentation is a function of total melanin present in the tissue, not of melanin content per melanocyte (Hu et al., 1997). Therefore, melanin content/culture was used as the main parameter for evaluating the effect of various neurotransmitters on melanogenesis.

The sympathetic nervous system employs neurotransmitters that influence adrenergic receptors (Table I). In the present studies, non-selective adrenergic agonist (epinephrine), non-selective β-adrenoceptor agonist (isoproterenol), and β_2-adrenoceptor agonists (salbutamol and metaproterenol) stimulated the growth and the melanogenesis of UM predominantly in cAMP-deleted medium, while α-adrenoceptor agonists (methoxanine and clonidine) and β_1- or β_3-adrenoceptor agonists (prenalterol and D-7114) showed no effect. These results indicate that adrenergic agonists mainly activate β_2-adrenoceptors and elevate the intracellular cAMP level. Phenylephrine, an adrenergic agonist, mainly activates α_1-adrenoceptors but also activates β-adrenoceptors at high concentrations, only stimulated the growth and melanogenesis of UM cultured with cAMP-deleted medium at high concentration. This indicates that the effect of phenylephrine on UM is also due to activation of β-adrenoceptors UM.

The parasympathetic nervous system utilizes neurotransmitters that influence cholinergic receptors (nicotinic and muscarinic receptors). Stimulation of muscarinic receptors inhibit the cAMP system or stimulate of phospholipase C. In the present studies, high concentrations of muscarine inhibited growth and melanogenesis of UM, possibly by inhibiting the cAMP system. There are five cloned human muscarinic receptors. The effect of various muscarinic receptor selective agonists on the UM will be studied in the future.

It has been reported that the effect of adrenergic neurons on iris pigmentation might be related to adrenergic α-receptors based on an experiment in rabbits (Odin and O'Donnell, 1982). However, in this rabbit study, only one representative α- and β-antagonist was tested. In the present studies, nine different adrenergic agonists with pronounced effects on all known sub-types of adrenergic receptors have been tested, and all experiments were conducted using four cell lines. The results were reproducible and the effects were consistent for all adrenergic agonists tested. Furthermore, our results are consistent with the results of the binding assays showing that β_2-adrenoceptors are the main adrenergic receptors in the human iris-ciliary body (Wax and Molinoff, 1987). Therefore, it seems likely that the release of neurotransmitters from adrenergic nerves associated with human UM mainly activates β_2-adrenoceptors.

The involvement of the β-adrenoceptor in melanogenesis may be regarded as unexpected, since α_1- and β_1-adrenoceptors are typically regarded as being associated with adrenergic neurotransmission (Bryan et al., 1981). This provides yet further evidence that the β_2-adrenoceptor is well represented in the human eye (Coakes and Shih, 1984; Nathanson, 1985; Jampel et al., 1987; Wax and Molinoff, 1987).

The mechanism of iris depigmentation at cell biology level in Horner's syndrome has never been satisfactorily explained. Our studies showed that cAMP-elevating agents is essential for the growth, survival and melanogenesis of UM. UM cultured with cAMP-deleted medium supplemented with isoproterenol could survive and produce melanin quite well. After deletion of isoproterenol, UM gradually lost viability and capacity for production of melanin, leading to reduced cell number and melanin content. It seems that adrenergic innervation plays an important role in the maintenance of integrity of UM. Paralysis of sympathetic nerve and deprivation of adrenergic neurotransmitters can lead to loss of UM and melanin, which is the underlying cause for the occurrence of iris depigmentation.

There are other diseases with iris depigmentation which may be related to the autonomic nervous system. For example, electron microscopy study of irides from Fuchs' heterochromic iridocyclitis patients have revealed a reduction of iridial melanocyte number and a decrease in the number and size of melanosomes, with accompanying degeneration of adrenergic nerves (Melamed et al., 1978; McCartney, Bull and Spalton, 1986). These findings indicated that the defect in melanogenesis in the UM may be due to abnormal adrenergic innervation, either primary or secondary to the inflammatory process (McCartney et al., 1986).

In summary, adrenergic agonists (mainly β_2-adrenergic agonists) stimulate growth and melanogenesis in UM, while cholinergic agonist has an inhibitory effect. This effect may be mediated by cAMP as the second messenger system. These studies indicate that homeostasis of UM may be maintained by the autonomic nervous system in vivo.

Acknowledgements

This work was supported by The New York Eye and Ear Infirmary Pathology Research Fund and the Department of Ophthalmology Research Fund.

References

Brittain, R. J., Farmer, J. B., Jack, D., Martin, L. E. and Simpson, W. T. (1968). Alpha-[(t-Butylamino) methyl]-4-hydroxy-m-xylene-alpha[1]-alpha[2]-diol (AH.3365): a selective β-adrenergic stimulant. *Nature* 219, 862–3.

Bryan, L. J., Cole, J. J., O'Donnel, S. R. and Wanstrall, J. C. (1981). A study designed to explore the hypothesis that beta-1 adrenoceptors are 'innervated' receptors and beta-2 adrenoceptors are 'hormonal' receptors. *J. Pharmacol. Exp. Ther.* 216, 395–400.

Cantacuzene, D., Kirk, K. L., McCulloh, D. H. and Creveling, C. R. (1979). Effect of fluorine substitution on the agonist specificity of norepinephrine. *Science* 204, 1217–9.

Coakes, R. L. and Shih, P. B. (1984). Effects of adrenergic drugs on aqueous humor dynamics in the normal human eye. 1. Salbutamol. *Br. J. Ophthalmol.* 68, 393–7.

Cook, N. A., Richardson, A. and Barnett, D. B. (1984). Comparison of the β_1-selective affinity of prenalterol and corwin demonstrated by radioligand binding. *Eur. J. Pharmacol.* 98, 407–12.

Eagle, R. C. (1988). Iris pigmentation and pigmented lesions: an ultrastructural study. *Trans. Am. Ophthalmol. Soc.* 84, 579–687.

Ehiger, B. (1966). Adrenergic nerves to the eye and to related structures in man and in the cynomolgus monkey (*Macaca irus*). *Invest. Ophthalmol.* 5, 42–52.

Emorine, L. J., Feve, B., Pairault, J., Briend-Sutren, M. M., Marullo, S., Delavier-Klutchko, C. and Strosberg, D. A. (1991). Structural basis for functional diversity of β_1-, β_2- and β_3-adrenergic receptors. *Biochem. Pharmacol.* 41, 853–9.

Feeney, L. and Hogan, M. J. (1961). Electron microscopy of the human choroid: II. The choroidal nerves. *Am. J. Ophthalmol.* 51, 1072/200–1083/211.

Hogan, M. J., Alvarado, J. A. and Weddell, J. E. (1971). *Histology of the Human Eye.* Pp. 209–55. P. 377. W. B. Saunders: Philadelphia.

Holloway, B. R., Howe, R., Rao, B. S., Stribling, D., Mayers, R. M., Briscoe, M. G. and Jackson, J. M. (1991). ICI D7114, a novel selective β-adrenoceptor agonist selectively stimulates brown rat and increases whole-body oxygen consumption. *Br. J. Pharmacol.* 104, 97–104.

Hu, D. N., McCormick, S. A., Orlow, S. J., Rosemblat, S. and Lin, A. Y. (1997). Regulation of melanogenesis by human uveal melanocytes in vitro. *Exp. Eye Res.* 64, 397–404.

Hu, D. N., McCormick, S. A., Orlow, S. J., Rosemblat, S., Lin, A. Y. and Wo, K. (1995). Melanogenesis in cultured human uveal melanocytes. *Invest. Ophthalmol. Vis. Sci.* 36, 931–8.

Hu, D. N., McCormick, S. A. and Ritch, R. (1993a). Studies of human uveal melanocytes in vitro: growth regulation of cultured human uveal melanocytes. *Invest. Ophthalmol. Vis. Sci.* 34, 2220–7.

Hu, D. N., McCormick, S. A., Ritch, R. and Pelton-Henrion, K. (1993b). Studies of human uveal melanocytes in vitro: isolation, purification and cultivation of human uveal melanocytes. *Invest. Ophthalmol. Vis. Sci.* 34, 2210–9.

Hu, D. N., Ritch, R., McCormick, S. A. and Pelton-Henrion, K. (1992). Isolation and cultivation of human iris pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* 33, 2443–53.

Imesch, P. D., Bindley, C. D., Khademian, Z., Ladd, B., Gangnon, R., Albert, D. M. and Wallow, I. H. L. (1996). Melanocytes and iris color: electron microscope findings. *Arch. Ophthalmol.* 114, 443–7.

Jampel, H. D., Lynch, M. G., Brown, R. H., Kuhar, M. J. and De Sovza, E. B. (1987). β-adrenergic receptors in human trabecular meshwork. *Invest. Ophthalmol. Vis. Sci.* 28, 772–9.

Lanier, S., Lafontan, M., Limbird, L. E. and Paris, H. (1996). Summary of the ASPIT-sponsored colloquium: alpha-2 adrenergic receptors: structure, function, and therapeutic implications. *J. Pharmacol. Exp. Ther.* 227, 10–16.

Laties, A. M. (1972). Specific neurohistology comes of age: a look back and a look forward. *Invest. Ophthalmol.* 11, 555–85.

Laties, A. M. (1974). Ocular melanin and the adrenergic innervation to the eye. *Trans. Am. Ophthalmol. Soc.* 72, 560–605.

McCartney, A. C. E., Bull, T. B. and Spalton, D. J. (1986). Fuch's heterochromic iridocyclitis: an electron microscopic study. *Trans. Ophthalmol. Soc. U.K.* 105, 324–9.

McCartney, A. C. E., Riordan-Eva, P., Howes, R. C. and Spalton, D. J. (1992). Horner's syndrome: an electron microscopic study of a human iris. *Br. J. Ophthalmol.* 76, 46–9.

Melamed, S., Lahav, M., Sandbank, U., Yassur, Y. and Ben-Sera, I. (1978). Fuch's heterochromic iridocyclitis: an electron microscopic study of the iris. *Invest. Ophthalmol. Vis. Sci.* 17, 1193–9.

Minneman, K. P. (1988). α_1-Adrenergic receptor subtypes, inositol phosphates, and sources of cell. *Pharmacol. Rev.* 40, 87–119.

Mukuno, K. and Witmer, R. (1977). Innervation of melanocytes in human iris: an electron microscopic study. *Graefes Arch. Clin. Exp. Ophthalmol.* 203, 1–8.

Mund, M. L., Rodrigues, M. M. and Fine, B. S. (1972). Light and electron microscopic observation on the pigmented layers of the developing human eye. *Am. J. Ophthalmol.* 73, 167–82.

Nathanson, J. A. (1985). Differential inhibition of beta adrenergic receptors in human and rabbit ciliary process and heart. *J. Pharmacol. Exp. Ther.* 232, 119–26.

Nimit, Y., Cantacuzene, D., Kirk, K. L., Creveling, C. R. and Daily, J. W. (1980). The binding of fluorocatecholamines to adrenergic and dopaminergic receptors in rat brain membranes. *Life Sci.* 27, 1577–85.

Odin, L. and O'Donnell, F. E. (1982). Adrenergic influence on iris stromal pigmentation; evidence for alpha-adrenergic receptors. *Invest. Ophthalmol. Vis. Sci.* 23, 528–30.

Prota, G. (1993). Regulatory mechanisms of melanogenesis: beyond the tyrosinase concept. *J. Invest. Dermatol.* 100, 156S–61S.

Ringvold, A. (1975). Electron microscopic study of the iris stroma in monkey and rabbit with particular reference to intercellular contacts and sympathetic innervation of anterior layer cells. *Exp. Eye Res.* 20, 349–65.

Scarpace, P. J. and Abrass, I. B. (1982). Desensitization of adenylate cyclase and down regulation of beta adrenergic receptors after in vivo administration of beta agonist. *J. Pharmacol. Exp. Ther.* 223, 327–31.

Tamm, E. R., Rohen, J. W., Schmidt, K., Robinson, J. C., Wallow, I. L. and Kaufman, P. L. (1997). Superior cervical ganglionectomy in monkeys: light and electron

microscopy of the anterior eye segment. *Exp. Eye Res.* 65, 31–43.

Wax, M. B. and Molinoff, P. B. (1987). Distribution and properties of β-adrenergic receptors in human iris-ciliary body. *Invest. Ophthalmol. Vis. Sci.* 28, 420–30.

Wilkerson, C. L., Syed, N. A., Fisher, M. R., Robinson, N. L., Wallow, H. L. and Albert, D. M. (1996). Melanocytes and iris color: light microscopic findings. *Arch. Ophthalmol.* **114**, 437–42.

Wolter, J. R. (1960). Nerves of the normal human choroid. *Arch. Ophthalmol.* 64, 120–4.

Zinn, K. M., Mockel-Pohl, S., Villanueva, V. and Furman, M. (1973). The fine structure of iris melanosomes in man. *Am. J. Ophthalmol.* 76, 721–9.